Federation Proceedings

VOLUME 10 *June 1951* NUMBER 2

NOTES ON THE THIRTY-FIFTH ANNUAL MEETING OF THE FEDERATION

Cleveland, Ohio, April 29–May 3, 1951

THE THIRTY-FIFTH ANNUAL MEETING of the Federation was held at Cleveland, Ohio, April 29–May 3. The Hotels Statler and Cleveland served as headquarters; scientific sessions were in the Cleveland Public Auditorium. Dr. Geoffrey Edsall, President of the American Association of Immunologists, was Chairman of the Executive Committee of the Federation and presided at the Joint Session held in the Public Auditorium on Monday evening.

Scientific sessions of the constituent Societies began Monday, April 30, and continued through Thursday afternoon, May 3. One hundred and twenty-six scientific sessions were held, at which 1298 papers were presented and 175 were read by title. Motion pictures were shown at a special session Tuesday evening. There was a Mixer and informal reception, arranged by the Local Committee, immediately following the Joint Session on Monday evening. Symposia were presented by the American Physiological Society, the American Society of Biological Chemists, the American Institute of Nutrition and the American Association of Immunologists. Three Societies had dinner meetings and various other groups arranged special functions such as business meetings, dinners and luncheons. The total registered attendance was 4787. Forty-nine industrial exhibits and 13 exhibits by members of the Federation were shown.

FEDERATION ACTIONS

The following actions of general interest were taken by the Executive Committee of the Federation:

1. Action on the proposed Constitution printed in the December 1950 issue of the *Proceedings* was deferred. By unanimous vote, ratified at the business meetings of the member Societies, Federation By-Laws 1, 2 and 8 were changed to provide for an 18-man Executive Committee with the Chairmanship passing in rotation among the Past-Presidents of the member Societies. A new committee will be appointed to consider further suggestions for a Federation Constitution and By-Laws.

2. The Federation assessment for the year July 1, 1951 through June 30, 1952 was set at $3 per member of each constituent Society.

3. Dr. M. O. Lee was reappointed Federation Secretary.

4. Plans were confirmed to hold the 1952 meeting in New York City April 14–18, and the 1953 meeting in Chicago April 6–10. The meeting will be held in Atlantic City in 1954 and on alternate years thereafter.

3. The American Physiological Society will be host Society for the 1952 meeting and will arrange the program for the Joint Session. Dr. D. B. Dill, as Past-President of the Physiology Society, will serve as Chairman of the Executive Committee beginning July 1, 1951.

6. A meeting of the Executive Committee of the Federation will be held in January 1952.

7. The subscription price of *Federation Proceedings* to non-members was raised to $6 per year, effective with Volume 11, 1952.

MILITARY UTILIZATION OF SCIENTISTS

The Federation is interested in the proper and best utilization by the armed forces of the scientific skills of its members who are serving temporarily. It is requested that members who enter active military service by enlistment, commission or from the reserves notify either the Secretary of their Society or the Federation Secretary for purposes of record. The Federation will attempt to obtain the correction of gross misassignment, or misutilization of skills of any members who request its help.

PLACEMENT SERVICE

Beginning with the February quarterly list of applicants, the lists of the Placement Service will be supplied on a subscription basis. Information as to rates will be distributed to those receiving this service. The registration fee for applicants will be increased to $3 annually.

CORRECTIONS AND ADDITIONS TO MARCH ISSUE

Page 34. DERN AND PULLMAN, *5th line from end of abstract:* The phrase "P = 0.1–0.02" should read "P = 0.01–0.02."

Page 52. The following abstract was omitted.

Studies on synergism of several anti-neoplastic agents. ABRAHAM GOLDIN, EZRA N. GREENSPAN AND EMANUEL B. SCHOENBACH. *Clin. Research Unit of Natl. Cancer Inst., U.S. Public Health Service, U. S. Marine Hosp., and Dept. of Preventive Medicine, Johns Hopkins Univ. School of Medicine, Baltimore, Md.*

Combinations of anti-neoplastic agents which had been shown to be effective when employed individually, were studied with respect to host survival time, inhibition of local tumor growth, and peripheral leukemic manifestations in dba mice moculated with a transplantable acute-stem-cell leukemia (Lymphoma 1210). The combinations studied included the following agents: 5-amino-7-hydroxy-1H-v-triazolo [d] pyrimidine (guanazolo; 8-azaguanine); 4-amino-pteroylglutamic acid (aminopterin); methyl bis (B-chloroethyl) amine (HN2); alpha-peltatin; and 2,4,6-triethyleneimino-s-triazine (TEM). Combined treatment with guanazolo plus aminopterin, guanazolo plus alpha-peltatin and aminopterin plus alpha-peltatin appeared to exert an additive inhibitory effect on the tumor without proportionate increase in host toxicity. Survival time was more prolonged than was observed when the drugs were employed individually. The other combinations of agents also affected the local growth and peripheral leukemic manifestations of the tumor to a greater degree, but the resultant host toxicity was increased to such extent that no prolongation of survival time was apparent.

Page 58. HAFT AND MIRSKY, *table:* The term "slide" should be changed to read "side."

Page 112. ROSTORFER, GEBER AND ONYETT, *line 15:* The sentence beginning in this line should read, "The nitrite-treated avian and mammalian cells reduced methemoglobin at the rates of 1.57 and 1.34 gm. %/hour, respectively." Delete the rest of the printed sentence.

Page 226. MORGAN AND GUEHRING, *line 1:* Change "pathogenic" to read "pantothenic."

Page 258. TAUBER, MCLEOD, GARSON AND MAGNUSON, *line 25:* The sentence beginning in this line should read, "The spirocheticidal substances were present in Precipitate III—1,2 associated with. . ."

Page 280. BEUTNER AND WILLIAMS, *line 10:* The word "invariably" should read "mostly."

Page 523. The following entry was omitted from the list of member exhibits.

Experimental radiographic visualization of the gallbladder. J. O. Hoppe, J. C. Seed* and J. W. Hart,* Sterling-Winthrop Research Institute, Rensselaer, N. Y. Radiopaque substances are used as diagnostic aids for visualizing soft tissue structures. A brief outline of some of the steps involved in the search for an improved cholecystographic medium as well as myelography and urography are illustrated. See also HOPPE, J. O. and S. ARCHER. *Federation Proc.* 10:310, 1951.

Retraction. The findings on holophosphorylase reported by *Irwin Feigin, Jerome Fredrick* and *Abner Wolf* in *Federation Proceedings*, Volume 9, page 170, 1950 and Volume 10, pages 181 and 184, 1951, are hereby withdrawn at the request of *Irwin Feigin* and *Abner Wolf*, since the results described cannot be duplicated.

JOINT SESSION OF THE FEDERATION

Cleveland, Ohio, April 30, 1951

Chairman: GEOFFREY EDSALL

PHYSIOLOGICAL ADAPTATION TO COLD IN ARCTIC AND TROPIC ANIMALS

LAURENCE IRVING

From the Arctic Health Research Center, Anchorage, Alaska

DURING recent years, and with the assistance of numerous colleagues, I have been carrying on studies of adaptations to arctic cold which might be described in physiological terms. As a result of these studies, we have developed an outline of the physiological economy of heat which appears useful as a guide for further investigation. Certain physiological systems are arranged in arctic animals for the conservation of heat; thus the metabolic costs of living in low temperature are apparently held down to about the same basal level of energy expenditure as is found in warmer climates. Observation of free wild arctic animals does not indicate that they expend more time feeding than do their relatives in warmer areas. In consequence they have time for the social, mating, play and exploratory activities which are equally essential in any climate for the preservation of a race.

I do not hesitate to call adaptive those devices possessed by arctic animals, and deficient in tropic forms, which obviously serve to conserve heat. Certain other physiological characters concerned with heat appear to be common to all climates; these are nonadaptive to cold and part of the common heritage of the warm-blooded condition.

We have recently measured the body temperature of arctic reindeer and dogs near the village of Barrow during a period in which the air temperature remained below −45° C. for a week. We have measured the rectal temperature of porcupines in the Talkeetna Mountains at temperatures around −30° C. These animals were living freely under natural conditions. In order to obviate the effects of disturbance during observation, the measurements were confirmed upon animals which were shot while at rest. We have gone to some trouble to ascertain that in the free condition the body temperature of arctic animals is, as reported for a few captives (1), characteristic of the warm-blooded state rather than of the climate in which they dwell. Body temperature is therefore not adapted to the conservation of heat.

In earlier studies (2) we reported that the resting metabolic rate of some arctic and tropic animals was proportional to their size, as is true among animals of temperate regions. The metabolic rates of all arctic and tropic animals examined fit closely upon the curve set by the equation

$$\text{Calories per day} = k \times \text{kg.}^{\frac{3}{4}}$$

The rates thus conform to the famous statement by Benedict of the relative metabolic rates of animals varying in size from the mouse to the elephant. We have found no indication that the basal metabolic rate is adaptable to cold, although I must reserve for future study some uncertainty about small arctic birds.

The length of the arctic animal's fur is conspicuous and its protective value against cold is shown by its effective use in Eskimo clothing. We have measured the insulation afforded by the fur of some arctic and tropic animals (3). In general, the insulating effect varies with the thickness of the fur. Among arctic animals the fur thickness increases with size up to that of the arctic white fox. In larger animals, such as the caribou and polar bear, no marked increase in thickness of fur is found. What further significance there may be in this interesting relation awaits study.

Knowing the factors of temperature and metabolic heat production, we can test the applica-

tion of the law of cooling, using a simple expression

$$\Delta TC = K \times I \times E$$

in which ΔTC is the largest temperature difference between the animal's body and the air which can be maintained at the basal metabolic rate, K is a constant for the units used, I is insulation and E is metabolic heat production. Plotting E against ΔTC gives lines representing $I \times E$ by their slope when all basal rates are referred to 100 per cent.

Applicable data are available for 9 arctic mammals and birds, 8 tropic mammals and birds along with comparable observations from other authors upon some 20 animals of more temperate regions. A plot of these observations brings out the basic system of animal heat conservation in all climates as a function of basal rate and insulation (1).

Insulation of adult arctic animals larger than the arctic fox is sufficient to enable them to maintain normal body temperature down to −30° C., the lowest attainable in these experiments. By theoretical indications and from observation of the animals' disregard for natural temperature around −50° C., it appears that arctic animals larger than the fox need not increase basal metabolic heat production until temperatures fall lower than −50° C., and then at only a small rate of increase.

Insulation is so meager among animals in the tropics that even the small diurnal and microclimatic changes often embarrass these animals with cold. But well-adjusted arctic animals do not shiver in any winter weather. In the wide range of temperature tolerable to them they seem to be in a better position with reference to the temperature of their climate than animals in the tropics.

This adaptive factor, insulation, is characterized by its physiological variability, for it may be suited to air temperatures of −50° C. or +30° C. It may promptly be changed to permit rapid dissipation of heat through the metabolism of violent exercise. It may obviously be altered morphologically during the seasons, although we have not yet measured this very apparent process.

It is also interesting to note that all three physiological functions — insulation, metabolic rate and body temperature—which are related in the application of the law of cooling as if they were single factors, are nevertheless obviously compounded of a variety of conditions which exist discretely at any one time in the animal body. Their formulation together then expresses the organization of the animal in administering the economy of metabolic heat.

We have recently made some measurements of the body skin temperature of arctic mammals and birds. At Barrow, we reported temperature of the body skin of 4 dogs in air at −3° C. to be from 33° C. to 37° C. In air at −19° C. the temperature of one dog's skin was 30° C.

This winter we were fortunate in being at Barrow Village during a period of low winter temperature. In air temperatures around −30° C. to −50° C., the rectal temperature of active or sleeping dogs was around 38° C. The surface skin temperature was around 30° C., with the immediately subcutaneous temperature a few degrees higher. Contrary to some views, these powerful arctic sled dogs are unusually stable toward those accustomed to their ways. We were able to make rectal and subcutaneous temperature measurements by inserting fine needles containing thermocouples even under the skin of the paws and nose without disturbing the animals.

The subcutaneous temperature of the bare foot pads was found variously to be at from +10° C. to +20° C. in air temperatures ranging from −30° C. to −45° C. Snow did not melt on the feet, but measurements on bare skin by surface contact are uncertain in such gradients.

Conditions for surface contact measurements on the bare muzzle are better and indicated low temperature. However, subcutaneous measurements on noses 2 or 3 mm. below the skin surface near the hair line at the muzzle registered only a little higher than those on the surface and were surprisingly low, indicating temperatures of 10° C. to 15° C. From the muzzle toward the eyes the subcutaneous temperature rose rapidly, reaching the body level on the head just back of the eyes.

With the assistance of Mr. Arnold Brower, we traveled by dog team to the location of a reindeer herd at Tapogaruk, about 70 miles south of Barrow. During the week the air temperature remained below −46° C. We were able to confirm that the temperature in the hooves and lower legs of both living and freshly shot deer remained regularly at 10° C. or even lower.

These observations epitomize, in our opinion, many others which we have made under less conclusive conditions upon the fleshy portions

of the exposed feet of arctic animals and birds, showing that they are often lastingly at low temperature. We have long been convinced that the skin of seals and other arctic marine mammals, and the feet of ducks and gulls must persistently remain at low temperature. We regard our measurements in the arctic only as a final demonstration of an *a priori* view that the exposed uninsulated tissues of many northern mammals and birds must for long periods exist at temperatures far below the level of the mammalian body.

That this low temperature will spare expenditure of heat is apparent, but it might be argued that as an unavoidable consequence of exposure, the condition is not an adaptation. We have usually considered that the properties of biological substances are set to operate well at normal mammalian body temperature levels. However, the cold state of some mammalian tissues as found in these studies imposes some possible difficulties in this concept. As substantiation of this, we have obtained, through the aid of Dr. Norman Abrahamsen, measurements of the melting point of fat from the marrow of the hind leg of a caribou shot near the Meade River in November. In the distal metatarsal bones the fat was soft, melting at 15° C., while in the upper femur, well within the body, it was 47° C. There are other indications that the fats in tissues exposed to low temperature have low melting points (4), and thus afford the apparent advantage of preserving flexibility in the cold.

I cannot forego the suggestion that certain tissues of warm-blooded animals in the arctic must have in this and other respects a sort of chemical adaptation of their substances to suit them for operation at temperatures lower than that of the mammalian body. In accepting this view, if it is reasonable, we will considerably modify the further experimentation by which we will search for physiological adaptations enabling animals to live successfully in arctic climates.

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BIOCHEMICAL MECHANISMS IN INSECT GROWTH AND METAMORPHOSIS

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From the Biological Laboratories, Harvard University, Cambridge, Massachusetts

Each of us . . . at the outset of his or her individual life story is microscopic and one sole cell. By that cell's multiplication and by its descendants' coherence, each of us attains . . . final form and size. The doings of this cell-assembly are . . . those of a being which is . . . a unity. Yet each of its constituent cells is a life centered in itself—managing itself, feeding and breathing for itself, separately born and destined separately to die. Further, it is a life helped by and in its turn helping the whole assembly, which latter is the corporate individual.

SIR CHARLES SHERRINGTON (1)

FOLLOWING Sherrington, we can recognize the growing organism as an expanding community of cellular lives, each centering in itself and yet each presupposing the whole. The growth and differentiation of this community takes place in a coordinated manner and according to a detailed blueprint inherited from the preceding generation. Indeed, genetics tells us that each cell is equipped with such a blueprint in its nucleus. A central problem of our science is to understand how the cells, as 'servants of the genes,' execute the hereditary plan of the final organism.

The phenomena here encountered are obviously not peculiar to the higher and more pretentious organisms such as the human species. In the tiger, as in the tiger beetle, growth, differentiation, and cellular coordination present substantially the same triad of problems.

The riddle of cellular differentiation seems no less baffling today than it did to preceding generations of biologists. We can only hope that the geneticist and the experimental embryologist, approaching the problem from opposite ends, may ultimately break through to one another. Most happily, however, significant progress has been made in understanding the two other aspects of the matter; namely, the mechanisms of cellular growth and intercellular coordination. Biochemistry with its new emphasis on synthetic processes is at long last gaining access to growth phenomena at the molecular level. Likewise, the physiologist is finding cellular events to be coordinated and controlled by chemical agents conveyed by blood or released from nerve.

It is from these biochemical and endocrinological points of view that I propose to consider what we have learned during the past few years about the coordination of growth and the growth process itself. The studies to be considered represent the joint effort of a group of investigators, chiefly graduate students, working at the Harvard Biological Laboratories. Insects were chosen as the subject of our study since the metamorphosis which punctuates their life history gives expression to morphogenic events which in most other animals are reserved to the early stages of embryonic development.

CECROPIA SILKWORM

Consider, for example, the Cecropia silkworm —an insect I shall use as a text for physiological thinking about the phenomenon of metamorphosis. Postembryonic development is here partitioned into three epochs: the growing larva, the quiescent pupa, and the reproducing adult. The transition between these successive stages is called metamorphosis.

The larva is fundamentally a digestive tract equipped with caterpillar tread. From the vegetation upon which it feeds, it acquires a stock pile of fat and protein which is stored away in a special tissue called 'fat body.' In a very real sense, the accumulation of this reservoir and stock pile is the biochemical objective of larval life. Thus the pupa, which forms within the larva during the pre-pupal period, is a closed system, save for the gradual evaporation of water and the interchange of respiratory gases. Likewise the moth, which forms within the pupa during the

[1] A number of studies considered in the present communication have been supported, in part, by the Lalor Foundation, the American Cancer Society, Inc., and the Public Health Service.

period of adult development, must also be built from the assets which the caterpillar has accumulated. For though the moth possesses a normal-looking digestive tract, it cannot be used because mouth parts are lacking at its anterior end. This paradox is answered by the finding that the 'normal-looking' digestive tract is devoid of digestive enzymes and therefore incapable of functioning (2). So, notwithstanding the fact that the larval, pupal, and adult insects are organized according to different plans, the three stages are, biochemically speaking, a reworking of the same old atoms and molecules.

ANATOMY OF METAMORPHOSIS

Metamorphosis begins at the end of larval life during the so-called prepupal period. The specialized structures of the caterpillar are at this time dismantled to make way for the equally specialized structures of the pupa. Dismantling of the one and construction of the other proceed side by side as the organism reshapes itself according to a new plan. These events are camouflaged by the old caterpillar skin which, like a loose sack, continues to cover the insect during the prepupal period. The shedding of this skin at the time of the pupal moult reveals the pupa itself.

Now the pupa is a stage of transition between larva and adult. It represents the first great stride in the metamorphosis of the species—a metamorphosis so profound that it amounts to the fabrication from the caterpillar of an essentially new organism, the adult moth.

If we look inside such a pupa, we find it filled with a great paired mass of fat body, the biochemical inheritance from the caterpillar. A more detailed search reveals the presence of a number of longitudinal structures: a dorsal heart and gonads, a ventral nervous system, and a central gut. Only in the skin of the pupa do we find any indication of the form of the future adult moth. Here the epithelium, but a cell's depth in thickness, shows a simple mapping-out of the moth's head, thorax, and abdomen. From this modest beginning within the pupa there is destined to develop the final adult insect, whose internal organization is no less complicated than that of a human being.

PUPAL DIAPAUSE

In the Cecropia silkworm as in many other insects, adult development does not proceed promptly after the pupa is formed. To the contrary, the rapid tempo of cellular events, which during a period of seven weeks has transformed the egg into the pupa, now comes to an abrupt halt. During the months that follow, the pupa persists in a state of developmental standstill, the pupal diapause.

For the insect in nature, the period of pupal dormancy provides for the overwintering of the species. Though the pupa makes no morphological advance during this period, the months of exposure to winter's low temperature are not 'time out.' By its direct action within the pupa, low temperature promotes certain endocrinological changes which assure the termination of diapause the following spring. Such a chilled pupa, after a few days of exposure to room temperature, shows an abrupt initiation of adult development signalling the termination of diapause. The construction of the moth within the pupa then proceeds with clock-like precision so that, at a temperature of 25°C., the adult insect emerges 21 days thereafter. It has taken ten months to develop that moth from the initial egg. And of the ten months, eight were assigned to the pupal diapause.

ENDOCRINE CONTROL OF DIAPAUSE

From the biochemical point of view, the most interesting aspect of metamorphosis is the pupal diapause. Here the biochemical mechanisms underlying normal growth and differentiation are mirrored in the abrupt transitions from growth to diapause and, with the latter's termination, back to growth. Since this sequence can be controlled at the convenience of the investigator, the matter becomes accessible to experimental analysis.

In view of the synchronization of morphogenic events at the cellular level, one fact is self-evident: there must be some overall control of the genesis and the termination of diapause. And it is no surprise to find this controlling mechanism to be endocrinological in character.

This fact is perhaps best illustrated by the behavior of the male sex cells of the dormant pupa when cultured *in vitro* (3, 4). If one removes the testis from a diapausing pupa and tears it open in a few drops of the insect's blood, an enormous number of little hollow cysts are released. The walls of each cyst are formed by a simple layer of undifferentiated cells, the primary spermatocytes. At the beginning of adult development each

of these cells undergoes two divisions (meiosis), followed by rapid differentiation into the spermatozoa of the moth.

When the dormant cysts of the pupal testis are placed in a simple hanging drop of the insect's blood, their behavior is remarkably conditioned by the status of the animal which donated that drop. In the blood of a diapausing pupa, the cysts show no significant development, though they survive for approximately a week (fig. 1*A*). In contrast, if one uses a drop of blood from a pupa that has just initiated adult development, the cells undergo prompt meiosis followed by differentiation into normal spermatids (fig. 1*B*).

The behavior of the spermatocytes in the hanging drop is therefore a kind of scale model of what goes on in the intact insect. During diapause the blood lacks a growth hormone which is necessary for the growth and differentiation of the testicular cells and of all other tissues. The secretion of this hormone in adequate titer terminates diapause and substitutes exuberant growth for developmental standstill. It seems proper to term this important growth factor the "growth and differentiation hormone" (5).

Time does not suffice to consider the role of this hormone in detail. Suffice it to say that growth at all stages in the life history seems to require its presence. It appears to be necessary for the growth and recurrent moulting of the larval insect, for the transformation of the larva into the pupa, and for the fabrication of the adult moth within the pupa. And, as we have seen, its absence following pupation results in the deficiency syndrome which we recognize as the pupal diapause.

As a growth factor of no mean proportions, the properties of this growth and differentiation hormone are of obvious interest. At long last this problem has become accessible by virtue of the assay method now provided by the tissue-culture technique. When tested in this manner, the hormone is found to behave as a protein or a smaller molecule tightly bound to a protein. It is nondialyzable, stable when heated to 75°C. for 5 minutes, but precipitated by exposure to 80°C. for 5 minutes (4). It has been impossible to mimic its action by any vertebrate hormone, vitamin, or biologically active substance.

The first visualization of the growth and differentiation hormone is presented in figure 2 (6). Six components are encountered in the electrophoretic pattern of active blood obtained from post-diapausing pupae. Figure 2*A* shows the still-active blood after exposure to 75°C. Figure 2*B* shows the inactive blood after heating to 80°C. We suspect that the small protein peak, present in *A* but absent in *B*, is attributable, in part, to the hormone itself.

In the intact insect the source of this growth and differentiation hormone has been identified. It is secreted by a pair of endocrine organs within the thorax, the 'prothoracic glands.' Curiously enough, these glands are able to secrete the hormone only under the tropic stimulation of a further hormone arising in 26 neurosecretory cells within the brain itself (7–12).

So within the insect we recognize the presence of a miniature endocrinological system—the prothoracic glands secreting the growth and differentiation hormone but only when triggered to do so by the brain hormone (fig. 3). Morphogenesis in this indirect manner is brought under the control of the brain, a rational device, since the brain then becomes the meeting place of the two great systems of integration, the nervous system and the endocrine system. Though the pupal diapause of the Cecropia silkworm results from the absence of the growth and differentiation hormone, it is noteworthy that the primary defect occurs at the level of brain function—the failure of the pupal brain to evoke adult development by triggering the function of the prothoracic glands (9, 13). The brain regains its secretory powers when exposed to the low temperatures of winter and, thus, the insect's life history is synchronized with the seasons (fig. 3).

ENZYMOLOGY OF METAMORPHOSIS

But the most interesting problem is still to be considered. Hormones are merely reagents. The reactants are the target organs, in this case the various tissues of the pupal insect. Between the hormonal reagent and the tissue reactants, a reaction must occur. And there is no reason why such a reaction should not be understandable in biochemical terms.

Now the simplest approach to the problem is diagrammed in figure 3. Let us assume that a biochemical defect is present in the cells of the diapausing pupa which prevents their metabolism from coupling to morphogenesis. This view would require the growth and differentiation hormone to promote some synthetic process which repairs the biochemical defect. I now propose to consider what evidence has been obtained as to the existence and identity of this hypothetical defect in the cells of the diapausing insect.

The first useful piece of information is the insensitivity of the diapausing pupa to cyanide. Though cyanide is one of our best insecticides, killing the caterpillar or the adult moth promptly, for the pupal insect it is not even a poison. Indeed, in our surgical maneuvers on pupae, we routinely use a Ringer's solution containing 0.01 M potassium cyanide. One can implant crystals of cyanide into a diapausing pupa without any ill effects. At the pH of the insect the agent is rapidly excreted through the tracheal system in the form of HCN. But before this occurs, its only obvious effect is to paralyze the intersegmental muscles of the abdomen. Attention therefore focusses on the status within the pupal insect of enzymes containing heavy metals and, more particularly, the iron-containing enzymes of the cytochrome system.

The curious insensitivity of the pupa to cyanide becomes intelligible when its tissues are studied spectroscopically. Under the Zeiss microspectroscope only the intersegmental muscles of the abdomen show the absorption bands of the three cytochromes, *b*, *c*, and $a + a_3$. Lacking these components, the pupal heart, for example, continues to beat normally when isolated in cyanide Ringer's. In the adult moth the situation is radically different. Here we find a well-developed cytochrome system in the vast majority of the insect's tissues. Evidently a remarkable synthesis of the cytochrome system takes place during adult development.

In order to appraise the matter in quantitative terms, cytochrome *c* was extracted from post-diapausing individuals and assayed spectrophotometrically (10, 12). Only a trace was found prior to the actual initiation of adult development. The termination of diapause and the progress of adult development were found to be accompanied by a rapid and progressive synthesis of cytochrome *c*.

Recognizing the difficulty of extracting the enzyme quantitatively, the study was repeated by manometric assay of cytochrome *c* in the brei of insects at various stages of development (14–16). The results were essentially identical with those previously demonstrated by the spectrophotometric method. There can be little doubt that the synthesis of cytochrome *c* begins its

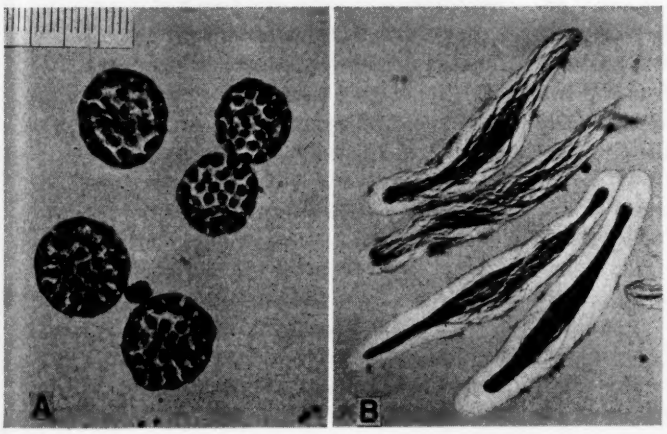

Fig. 1. CYSTS OF DORMANT PUPAL spermatocytes in hanging-drop cultures. *A:* The cysts have been cultured in inactive blood from a diapausing pupa and show no development. *B:* The cysts have been cultured in a drop of active blood from a postdiapausing insect. The growth and differentiation hormone has caused each spermatocyte to undergo meiosis and prompt differentiation. Each cyst is thereby transformed into an elongate bundle of spermatids. Smallest division on scale equals 10 μ.

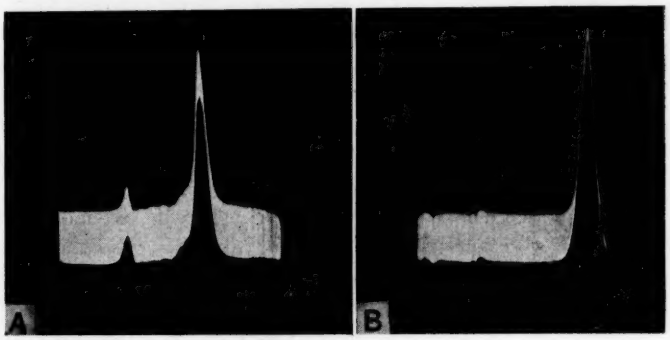

Fig. 2. ELECTROPHORETIC patterns of dialyzed blood from post-diapausing pupae. *A:* The still-active blood after heating for 5 minutes at 75°C. *B:* Blood inactivated by 5 minutes' exposure to 80°C. The small peak, present in *A* and absent in *B*, is thought to consist in part of the growth and differentiation hormone.

synchrony with the termination of diapause and continues in step with the progress of adult development.

Cytochrome *b* and cytochrome oxidase were found to behave somewhat differently. Indeed the initiation of adult development is accompanied by a precipitous *decrease* in the activities of these enzymes. In the case of cytochrome oxidase, the initial decrease is followed by a rapid synthesis which begins on approximately the second day of development (12, 14). To the contrary, the low activity of succindehydrogenase presumably mirroring the function of cytochrome *b*, persists throughout the first half of adult development and only thereafter undergoes rapid increase (17). These changes in cytochrome *b* resemble in all respects the behavior of the two dehydrogenases we have studied; namely, malic dehydrogenase and α-glycerophosphate dehydrogenase. A resemblance is also evident between these results and those obtained by Agrell (18, 19).

Consequently, the activation of the growth mechanism at the outset of adult development and the sudden release of mitotic activity show a positive correlation with the behavior of cytochrome *c* and a negative correlation with the changes occurring in cytochrome *b*, cytochrome oxidase, and the two dehydrogenases that we have studied.

In the absence of the cytochrome system, the pupal insect is still able to consume oxygen apparently by the use of a flavoprotein as terminal oxidase. Biological assay of protein-bound riboflavin demonstrates a maximal titer of flavoprotein during diapause and a progressive decrease following the onset of adult development (14, 16). Since the autoxidation of flavoprotein produces hydrogen peroxide rather than water, it is noteworthy that the changes which it undergoes are paralleled by those occurring in catalase.

From these enzymatic studies we learn that the diapausing pupa, with the exception of its abdominal muscles, is characterized by a cyanide-stable flavoprotein functioning as terminal oxidase. At the outset of adult development this system begins to break down and is replaced by a cytochrome system containing an increasing titer of cytochrome *c* and a temporarily decreasing titer of cytochrome oxidase. Thus the termination of diapause is accompanied by profound alterations in two of the three great electron transmitters—flavoprotein and cytochrome. In the absence of the cytochrome mechanism, the flavoprotein system can apparently provide for maintenance, but not for mitotic activity or that forward progress which we recognize as growth. The coupling of growth to metabolism appears to be synchronized with the synthesis of the cytochrome mechanism.

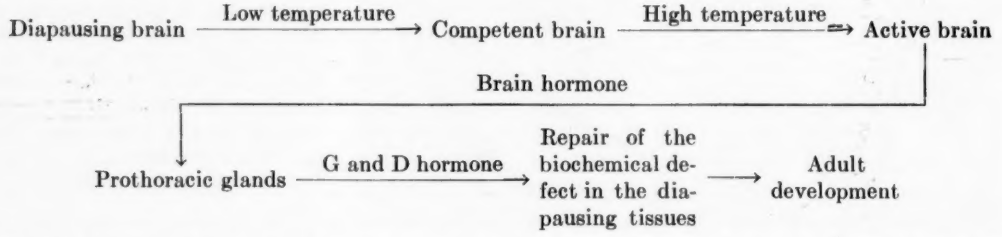

Fig. 3. DIAGRAM of endocrine control of adult development by the brain and prothoracic glands. For explanation, see text.

Our search for a biochemical defect in the diapausing insect has therefore revealed at least one such defect in terms of the absence of a functional cytochrome system. Is it possible that the cytochrome mechanism plays some special role in morphogenesis and that the absence of this system is the fundamental biochemical defect in the diapausing cells? I shall finally consider evidence that tends to answer this question in the affirmative.

If the function of the cytochrome system is prerequisite for morphogenesis, then growth should be brought to a standstill by inactivation of this enzyme system. Fortunately, in an animal such as an insect, where hemoglobin is absent and where environmental gases pass directly to the tissues through the tracheal system, it is possible to block the function of the cytochrome system by a highly specific agent, carbon monoxide. Only one difficulty is encountered: the combination of cytochrome oxidase with carbon monoxide is reversed by oxygen. The ratio between the ten-

sions of carbon monoxide and oxygen must therefore be high; in any case not less than about 20 to 1.

We have used two means to accomplish this end. In the first method the animals were placed in air-filled chambers and compressed with 3 to 10 atmospheres of carbon monoxide. In this manner the oxygen tension was held constant and the carbon monoxide tension and total pressure increased. In the second method the experiment was performed at a total pressure of one atmosphere, the animals being placed in a chamber and exposed to a mixture of 5 per cent oxygen and 95 per cent carbon monoxide. Thus the total pressure was held constant and the oxygen and carbon monoxide tensions varied.

Substantially the same results were obtained by the two methods (17, 20). Diapausing pupae were unaffected. Pupae just prior to the initiation of development remained alive but underwent no development during the experimental period of 21 days. In the case of animals that had already escaped from diapause, the carbon monoxide caused a complete or almost complete cessation of development and enforced an artificial diapause which persisted during the experimental period of 21 days. When returned to air such animals usually recovered promptly and resumed development where they had left off.

Results of this character consistently observed during the past three years, strongly suggest that the cytochrome system plays a crucial role in the metabolism of morphogenesis (21). We find this to be true, not only in the Cecropia silkworm, but also in the several other insects which we have examined, for example in the adult development of the blowfly *Phormia* and the parasitic wasp *Mormoniella*. Indeed, by exploiting the transparency of *Mormoniella*, it has been possible to demonstrate a partial reversibility by light of carbon monoxide's inhibition of growth (17). This result confirms a previous finding on *Drosophila* (22) and gives assurance that carbon monoxide's power to dissociate growth from maintenance results from its combination with cytochrome oxidase.

Among several hundred chemicals which we have tested, selective blockade of the growth mechanism has been accomplished by two further agents—diphtheria toxin and certain imidazole derivatives (23).

Injection of 0.1 to 1 *gamma* of diphtheria toxin into mature caterpillars or post-diapausing pupae causes prompt cessation of development followed by delayed death about a week thereafter. Diapausing pupae, however, survive 1000 times this dose of toxin. Its only visible effect is to render the intersegmental muscles of the abdomen flaccid and uncontractile.

These results become intelligible in terms of Pappenheimer's (24) previous studies where diphtheria toxin was found to correspond apparently to the free protein moiety of the diphtheria bacillus' cytochrome *b*. Its astonishing toxicity for the mammal is thought to result from its functional derangement of the cytochrome system.

Consequently, within the diapausing insect, the toxin finds no mechanism of action, save in the abdominal muscles, the only tissue possessing an intact cytochrome system. However, in the growing larva and developing adult, the reaction between the toxin and the cytochrome system causes a prompt cessation of growth.

It is possible to accomplish this same end by the use of imidazole and certain of its derivatives; namely, pilocarpine, dimethyl imidazole, and methyl imidazole (23). These substances are distinguished by their remarkable affinity for oxidized heme (hematin) with which they combine to form a stable hemichrome (25). Evidently, within the living insect they act as scavengers of hematin and render this important molecule unavailable for the synthesis of cytochrome. Though the pupal insect continues to live for a year or longer after the injection of pilocarpine, the onset of adult development is permanently prevented. In insects that have already terminated diapause and initiated the synthesis of the cytochrome system, pilocarpine is still capable of blocking development, though the threshold is now twenty times higher than that of the pupa. Developing animals blocked in this manner undergo delayed death several weeks following injection.

Summing up the information available at the present time, we find that the growth mechanism can be dissociated from the metabolism of maintenance by the action of several substances whose biochemical target within the living cell is the cytochrome system. As Needham (26) has prophetically remarked, "It almost looks, therefore, as if non-ferrous respiration cannot be geared to morphogenesis".

Our initial objective of understanding the biochemistry of insect growth and metamorphosis has therefore led us far afield—to a meeting place between energy and synthetic mechanism at the subcellular level. But as far as our initial and

humbler objective is concerned we feel assured that the synthesis of the cytochrome system from its hemochromogen precursors is causally related to the termination of pupal diapause. We are therefore persuaded to regard the absence of the cytochrome system as a basic biochemical defect in the cells of the diapausing insect. Since the repair of this biochemical defect, as we have seen, is brought to pass by the 'growth and differentiation hormone,' the way seems open to an analysis of this endocrinological reaction in biochemical terms.

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AMERICAN ASSOCIATION OF IMMUNOLOGISTS

Symposium on Labelled Antigens and Antibodies[1]

Chairman: MICHAEL HEIDELBERGER

FATE OF I^{131} LABELLED BOVINE GAMMA GLOBULIN IN RABBITS*

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THE TECHNIQUE of labelling and tracing protein antigens with radioactive iodine is being used increasingly in immunologic studies. Mention of a few of the more important chemical, physiological, and immunological features of this technique will indicate both its possibilities and its limitations. I^{131} can be added to almost any protein in traceable amounts without altering the protein immunologically. Accurate quantitation of I^{131} in blood, tissues, or immunologic precipitates is possible with a Geiger counter, using proper absorption and coincidence corrections. These radioactivity measurements indicate the concentration of antigen in the material tested. In addition, approximate histologic localization of the I^{131} antigen can be obtained with autoradiographs, in which ordinary histologic sections are mounted on special photographic emulsions and, in effect, take a picture of their own radioactivity. Autoradiographs do not, however, permit the detailed histologic localization possible with fluorescent antibody (1) or dye-labelled proteins (2, 3).

Protein antigens labelled with I^{131} appear to retain the I^{131} label *in vitro* (4) and *in vivo* (5) as long as they are immunologically active. The studies of Knox and Endicott (5), as well as our own observations, show close agreement between quantitative precipitin and radioactivity determinations of antigen.

Catabolism of I^{131} protein antigens liberates non-protein-bound I^{131}, and in animals prefed ordinary iodide, this liberated I^{131} is rapidly eliminated, primarily in the urine with lesser amounts in breath and feces. Following injection of an I^{131} antigen, the radioactivity in blood and tissues can be separated into protein-bound and non-protein-bound fractions; the protein-bound activity represents residual antigen while the non-protein-bound I^{131} present in the blood and tissues, plus that excreted in the urine, provides a reliable index of the rate of antigen catabolism. This technique, therefore, gives an excellent quantitative measure of antigen concentration in blood and tissues and also indicates rate of antigen catabolism.

Before discussing our observations on the rabbit's handling of the antigen, I^{131}-labelled bovine gamma globulin (I*-BGG), let us look first at the rabbit's metabolism of his own plasma globulin. Madden and Whipple (6) have shown that plasma proteins exist in dynamic equilibrium in vascular and extravascular fluids and serve to supply, in part, the nutritional needs of the body's tissues. Our findings using homologous globulin labelled with I^{131} (I*-RG) concurred with their's. Figure 1, which is a semilogarithmic plot of the labelled globulin content of blood against time in days, shows that within one day after intravenous injection there was a sharp drop in the I*-RG blood content to approximately one-third the amount injected. This drop represents the equilibration of I*-RG between vascular and extravascular components of the plasma protein pool and indicates twice as much globulin in extravascular as in vascular fluid. Once the equilibrium was established, the I*-RG disappeared at a constant rate with a half life of about 2 days,

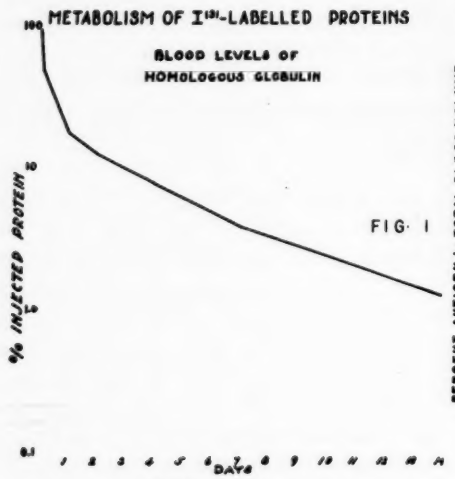
[1] May 1, 1951, Cleveland, Ohio.

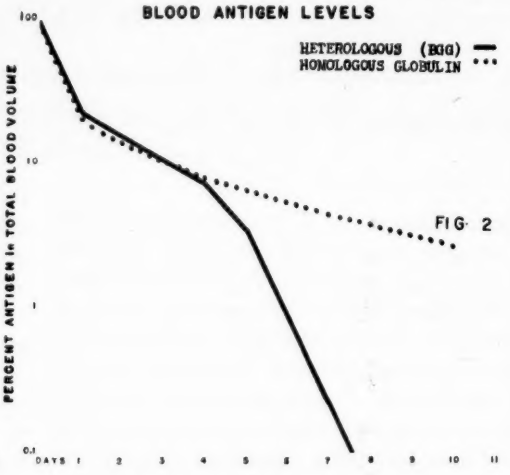
* This work was supported by grants from the Atomic Energy Commission and the United States Public Health Service.

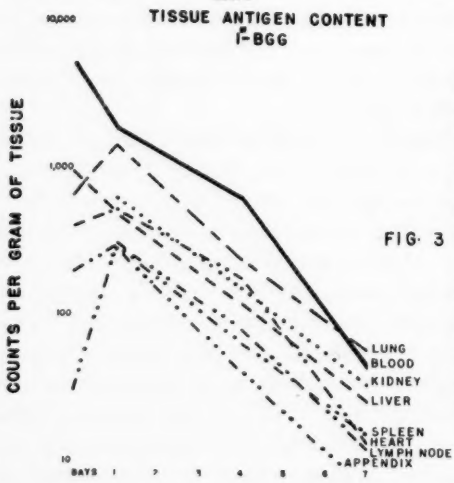
representing the rate of catabolism of homologous globulin.

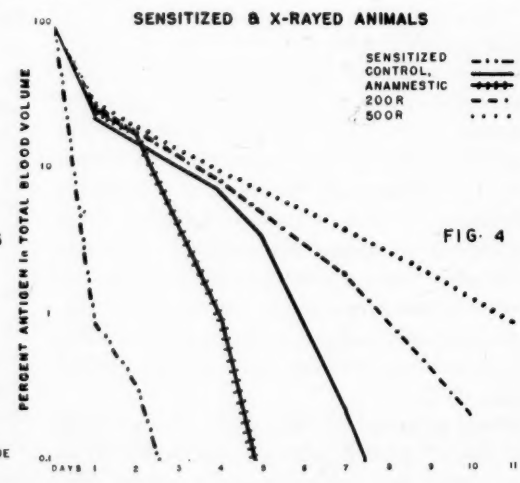
I*-BGG injected intravenously into rabbits in doses varying from 1 to 500 mg. was handled similarly to the I*-RG for the first 4 days (7). The second graph of blood content of labelled protein shows again the disappearance curve for homologous globulin. In addition, the solid line represents the amount of I*-BGG in the blood following a 75-mg. injection and shows an equilibration period followed by a 3-day period of catabolism similar to that for the homologous protein. It appears that this antigenic, heterologous protein equilibrates between vascular and extravascular fluids just as does homologous protein. In so doing, the antigen, no doubt, comes into intimate contact with most or all of the body's tissues. The catabolism of heterologous globulin at a rate almost identical with that of homologous globulin for 4 days would further suggest that in this initial period the body utilizes the heterologous globulin similarly to its own globulin.

After the fourth day, the disappearance rate of I*-BGG from the blood increased sharply. It is most likely that this increased elimination rate is caused by the appearance of antibody which, however, could not be detected by our complement-fixation technique[2] (8) until all antigen had









Figs. 1 to 4

[2] All antibody determinations were done by the complement-fixation technique described by Kent *et al.* This method employs a dilution of antigen (1:500,000) which is optimal for the detection of minimal amounts of antibody with four 50 per cent hemolytic units of complement. The minimal amounts of antibody as detected are of the order of 10 μg. antibody nitrogen per cc. of serum.

been eliminated, 7 days following injection. The first antibody to appear probably combines with antigen in extravascular or intravascular fluids and the combination is rapidly eliminated from the circulating fluids and catabolized, as will be discussed later. Since this is accomplished without measurable accumulation of antigen in any organ or tissue, it is evident that the breakdown of antigen is an extremely rapid process and/or one widespread throughout the body.

The elimination of I*-BGG from the various tissues approximately paralleled its elimination from blood, as would be expected with a dynamic equilibrium of the antigen in the body. In figure 3 the amount of radioactivity per gram of tissue or blood following an intravenous injection of 75 mg. I*-BGG is plotted against time in days. The activity concentration was higher in blood than in any tissue, and disappearance rates were, in general, parallel with a slight lag in the disappearance of radioactivity from the tissues after the fourth day. All radioactivity levels were insignificant after the seventh day, with no tissue showing measurable retention of antigen. Complement-fixation tests for antigen in the tissues after the seventh day were also negative.

Detectable circulating antibody appeared on the seventh day after virtually all antigen had disappeared. This is in keeping with the findings of Hawn and Janeway, who found disappearance of BGG and appearance of antibody between one and two weeks following injection of 2 gm. of antigen (9). Following injection of 75 mg. of I*-BGG, the peak antibody level occurred on the tenth day and all detectable circulating antibody disappeared within approximately 20 days after antigen injection. It is apparent that the first antibody is produced in the presence of antigen, but that the major part of antibody production goes on in the complete absence of antigen.

We found considerable support for the view that the increased antigen disappearance rate seen after 4 days in I*-BGG injected control rabbits is caused by appearance of antibody. Figure 4 compares blood antigen levels in 5 experimental groups of rabbits, each with a different immune response: actively sensitized rabbits with detectable circulating antibody present at the time of injection of antigen showed rapid disappearance of antigen immediately; previously sensitized animals without detectable circulating antibody showed a rapid antigen disappearance beginning 2 days after injection, in keeping with an accelerated, anamnestic response; animals receiving 200 r whole-body x-radiation showed delay in and decrease of rapid antigen elimination, corresponding to the inhibition of the immune response resulting from the radiation; animals receiving 500 r x-radiation showed neither rapid antigen elimination nor any immune response. The appearance of detectable serum antibody in these 5 groups of rabbits always followed immediately upon the rapid disappearance of antigen, as seen in figure 5. Here the 5 antigen disappearance curves are given in temporal relation to the appearance of complement-fixing antibody, indicated by the black bars. In the first 4 instances, rapid antigen disappearance was closely followed by appearance of detectable antibody, while after 500 r neither rapid antigen elimination nor detectable antibody was seen.

Further support for the relationship of antibody to rapid elimination of antigen was found after injection of passive antibody into rabbits

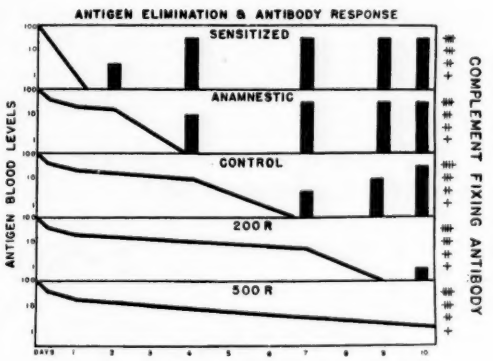
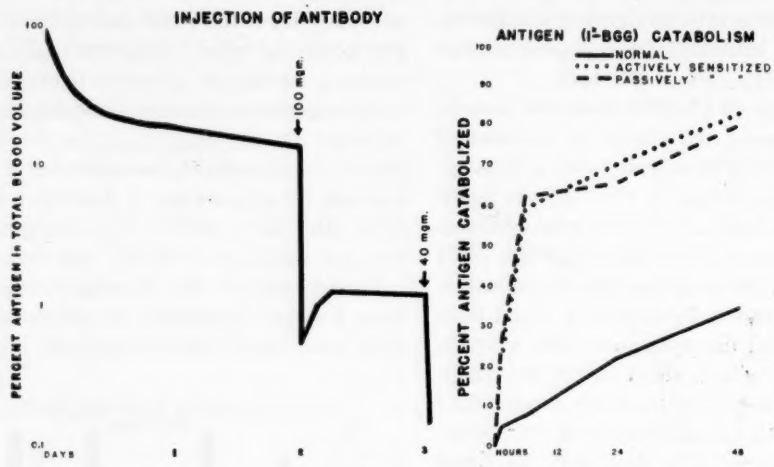


Fig. 5

with circulating I*-BGG. In figure 6 the I*-BGG concentration in blood is plotted against an expanded time scale. The injection of passive antibody, 100 mg. on second day and 40 mg. on third day, caused a prompt drop in blood antigen content. The rise in blood antigen level shortly after the first injection represents the re-establishment of equilibrium of antigen between the depleted vascular fluid and the antigen-containing extravascular fluid.

On the basis of these interrelationships between antigen disappearance rate (ADR) and appearance of antibody, we have used the mathematical expression of ADR as an index of immune response (10). This measure is extremely sensitive and will reveal an immune response which may produce only enough antibody to combine with the antigen present, and not enough to cause a detectable excess in the serum. Such weak antibody responses may occur after moderate

doses of x-ray or following extremely small doses of antigen and result in rapid antigen elimination without detectable antibody. Later demonstration of a specific anamnestic response indicates that such animals have made a previous immune response to the given antigen.



Figs. 6 (*left*) and 7 (*right*)

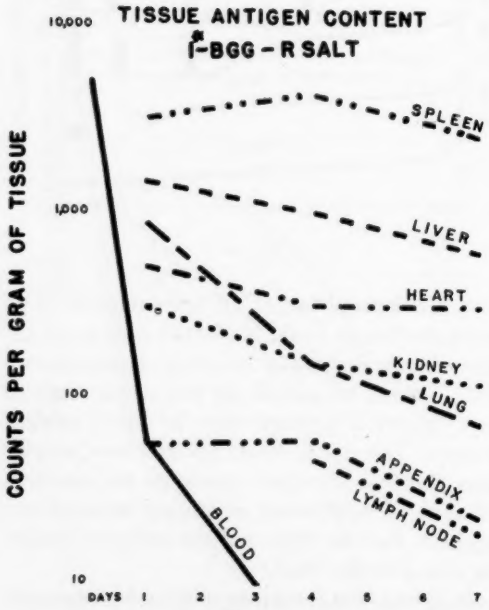


Fig. 8

The mechanism of rapid elimination of the antigen-antibody combination from the blood and the associated rapid antigen catabolism are not completely understood. It is most likely that antigen-antibody complexes would be taken up by phagocytic cells, thus accounting for the rapid removal of antigen from the blood. In investigating antigen catabolism, we attempted to find whether the increased rate of catabolism in sensitized animals could be accounted for alone by presence of antibody, and therefore, presumably by increased phagocytosis or whether there was, also, a cellular enzymatic adaptation associated with the process of sensitization, which facilitated more rapid antigen catabolism (11). To do this, we compared the rates of I*-BGG catabolism in actively sensitized rabbits and in control rabbits given 95 mg. of passive antibody, an amount calculated to be approximately equal to the amount in those actively sensitized. Figure 7 shows the rates of I*-BGG catabolism, as measured by non-protein bound I^{131} in urine, blood, and tissues in actively sensitized, passively sensitized, and control rabbits for the first 48 hours after intravenous injection. The rates of antigen catabolism for actively and passively sensitized rabbits were almost identical. The antigen disappearance rates in actively and passively sensitized groups were also similar. It appears, therefore, that both the increased rate of disappearance of I*-BGG from the blood and the rapid catabolism found in sensitized animals can be accounted for by the presence of antibody alone. Whether the sensitized animal makes any physiologic or enzymatic adaptation to an antigen such as I*-BGG other than the production of specific antibody cannot be definitely stated at this time. However, there is nothing that happens in the immune catabolism of I*-BGG that cannot be duplicated in a control animal by addition of passive antibody.

These findings agree with the antigen tracer study of human globulin using the fluorescent antibody technique (1). There is, however, a marked discrepancy between our findings and those obtained by workers using BGG or similar proteins labelled with various azo dyes (3). The azo dye-labelled proteins were found concentrated and retained for long periods of time in the reticulo-endothelial system. In an effort to explain these differences, we attached to I*-BGG by azo conjugation one of two compounds, R salt (12, 13) or para-aminobenzoic acid, and traced these doubly labelled antigens by both color and radioactivity. Using these antigens, we found an uptake and retention of antigen by the reticulo-endothelial system, just as described by those using the various azo dye-labelled proteins. Figure 8 indicates azo-I*-BGG concentration in blood and tissues following intravenous injection of 75 mg. of the antigen. The blood level fell rapidly while tissue concentrations, especially in those tissues rich in reticulo-endothelial cells, dropped very slowly. The tissue retention of azo-I*-BGG is in marked contrast to the findings with I*-BGG where no tissue antigen retention occurred (fig. 3). Thus, it appears that azo labelling markedly changes the *in vivo* behavior of protein antigens such as BGG.

SUMMARY

Tracing of the antigen I*-BGG in rabbits indicates that the antigen is handled much like the animal's own globulin until antibodies to it are formed. Before the appearance of antibodies, the antigen exists in equilibrium between vascular and extravascular fluids, has free access to the tissues of the body, and is neither concentrated nor retained by any tissue or organ. With the appearance of antibody, the antigen is rapidly removed from blood and extravascular fluids, probably by phagocytic cells and is rapidly catabolized. There does not appear to be any cellular enzymatic adaptation necessary in the sensitized, rapid catabolism of antigen. Antibody production, although initiated in the presence of antigen, is carried on long after all antigen has disappeared.

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FLUORESCENT ANTIBODIES AS HISTOCHEMICAL TOOLS[1]

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THE ANTIBODY molecule can be conjugated with fluorescent compounds without apparent loss of specificity (1–3). This fact makes it possible to employ the precipitin reaction as a method for the histochemical detection of small quantities of material in tissue cells. The only basic requirement for success is that the material sought be sufficiently antigenic so that the requisite antiserum can be produced.

Purified antisera were chemically conjugated with fluorescein isocyanate to produce a brilliantly fluorescent protein solution in which the antibody molecules as well as the other protein molecules are fluorescent. When such a fluorescent antibody solution is allowed to react with organisms or tissue cells containing homologous antigen, some of the antibody is precipitated over those areas where the antigen is located. The excess of the fluorescent solution can be washed away and the deposited antibody visualized under the fluorescence microscope. This reaction has so far been successfully employed to trace the fate of injected bacterial polysaccharides and foreign animal proteins after injection into mice, and to localize viral and rickettsial antigens in infected cells. In the brief time available, I will summarize the successes which we have so far had in the use of this method, list the failures and point out some of the difficulties.

When foreign substances such as bacterial polysaccharides (4) or animal proteins (5) are injected intravenously into mice, they rapidly begin to leave the blood stream and make their appearance in the connective tissue throughout the animal. They are also found in high concentration in all the phagocytic cells in the body. They can also be found in a low but distinct concentration in lymphocytes in the nodules of the spleen and lymph nodes. In general, the epithelial cells of the animal are well protected against the entrance of these antigenic materials but they all made an appearance in the hepatic cells and in the endothelium of the renal tubules. There are minor differences in their distribution which there is no time to describe.

The most striking difference among the antigens so far studied is their rate of disappearance. Crystalline egg albumin (dose 10 mg.) disappeared within a few hours. Crystalline bovine serum albumin persisted for about 2 days. Human plasma gamma globulin (dose 4 mg.) persisted for a week. It is apparent that the rate of disappearance is inversely proportional in a rough way to the molecular size of the antigen. In great contrast to this rather rapid disappearance of protein antigens, the capsular polysaccharides of pneumococcus type II, pneumococcus type III, and Friedländer bacillus type B, injected in comparable doses, persisted for weeks to months. In the case of all six of these antigens, the connective tissue and the phagocytic cells of the reticulo-endothelial system contained antigen after it had disappeared from other locations.

An unexpected finding was that the protein antigens, or fragments of them, could regularly be found in the nuclei of all the cells into which they penetrated. This confirms the data of Crampton and Haurowitz, who found radioactivity in the nuclear fractions of liver homogenates after the injection of proteins labelled with I^{131} (6).

The method is not only useful in tracing injected foreign proteins or polysaccharides; its utility has already been demonstrated in the study of cells infected with rickettsiae and with viruses (7). For example, the cells of the monkey parotid infected with mumps virus can be readily visualized by use of labelled antibody against the mumps virus. Similarly, the rickettsiae of epidemic typhus and Rocky Mountain spotted fever have been found in infected cells in the liver and spleen of the cotton rat and in smears of the gastrointestinal tract of individual infected lice. An extensive study is now being carried out by Dr. Barbara Watson of the progress of mumps infection in the embryonated hen's egg. Two other viruses with which preliminary experiments have been successful are

[1] Aided by grants from the Life Insurance Medical Research Fund, the Helen Hay Whitney Foundation and the Eugene Higgins Trust.

influenza A virus in the infected mouse lung and in the infected hen's egg embryo, and the virus of canine hepatitis in the organs of infected dogs. Work with the hepatitis virus is being carried out in collaboration with Dr. D. L. Coffin.

Attempts to perform the same sort of experiments with neurotropic viruses have so far failed. Although it was possible to demonstrate mumps virus in the brain of a monkey with experimental parotitis but without neurological symptoms (8), similar attempts with the Columbia strain of SK virus and Theiler's virus GDVII have been unsuccessful. Attempts to find the Lansing strain of poliomyelitis virus in infected monkey cord and brain and in tissue cultures from human skin and brain have so far failed (9).

The major pitfall in the application of this method is the very regular appearance of what we have hitherto called 'non-specific staining' (3). It is already becoming clear that much of this non-specific staining is the result of so-called natural antibody, and due to the possession of common antigens by animal tissues of different species and bacteria with which the serum donors are frequently infected. So far in our studies, we have avoided this non-specific staining by absorbing our conjugated antisera with normal tissue from animals whose tissues it was proposed to study. But it is probable that this method is only successful when the normal tissue used for absorption contains appreciable amounts of the antigen belonging to the interfering antigen-antibody system. Much remains still to be worked out with regard to such cross reactions.

Despite this difficulty, the use of fluorescein-labelled antibody as a specific histochemical reagent has already proved to be a useful tool in the localization of antigenic materials in tissue cells. It has the disadvantage inherent in other histochemical methods in that it is difficult to quantitate, particularly so because the amount of light coming from any given cell is very small. Its advantage lies in its apparent high sensitivity, and in its specificity.

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IMMUNOCHEMICAL STUDIES WITH LABELLED ANTIGENS[1]

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PREVIOUS experiments with arsanil-azo-proteins had demonstrated that most of the antigen injected into rabbits disappears rapidly from the blood and passes into liver, spleen, bone marrow and lung (1). Similar results are obtained with iodo-ovalbumin tagged with I^{131} (2). Table 1 shows the approximate activity of various organs at different times after the injection of iodinated ovalbumin.

It is apparent from the figures recorded in table 1 that the antigen content of the organs decreases rapidly during the first hours after injection of the antigen, but that the decrease thereafter is very slow. At the end of 29 days the liver still contains 0.018 per cent of the injected activity. If this were present as unchanged iodo-ovalbumin, it would correspond to 15.5 μg. or 2×10^{14} molecules of iodo-ovalbumin in 62 gm. of liver. Since the volume of a liver cell is about 10^{-9} to 10^{-8} ml., each cell would contain approximately 10,000 antigen molecules at the end of the 29th day. Results similar to those recorded in table 1 were obtained when the amount of iodo-ovalbumin injected was reduced to 9 to 11 mg. or when the iodo-ovalbumin was replaced by iodinated beef serum globulin.

In a series of experiments we injected in rabbits 2 to 5 doses of iodo-ovalbumin in intervals of 3 days. Only the last of these injections was labelled by I^{131}. The rabbits were killed 24 hours after the last injection. The distribution of the activity in the organs and in the liver fractions was the same as that which followed a single injection of I^{131}-iodo-ovalbumin.

The most interesting result of our experiments is the finding that the injected antigen is rapidly incorporated into the small granules (microsomes) and that it appears shortly thereafter in the large granules (mitochondria) where it remains for long periods of time. The separation of the granules from nuclei and from the supernatant solution (table 1) was achieved by fractional centrifugation (2, 3). The yields of nuclei, mitochondria and microsomes were about 20 per cent of the total liver homogenate for each of these fractions, while the supernatant solution formed 40 per cent of the homogenate. All four fractions were precipitated by trichloroacetic acid and the precipitated proteins washed with acetone and ethanol. Table 1 shows the relative activities of the washed proteins of the subcellular fractions. When radio-iodinated ovalbumin was added to a normal rabbit liver homogenate under the conditions used in our procedure, only traces were adsorbed to the granules, while the bulk was found in the supernatant solution. This proves that incorporation of the antigen into the granules occurs *in vivo* and not during the fractionation. The finding of the intravenously injected antigen in cellular granules indicates that it passes rapidly through the membranes of the liver cells. Since the molecules of ovalbumin and beef serum globulin are macromolecules which are not able to pass through semipermeable membranes, we have to assume that their incorporation into the liver cells occurs by some process akin to phagocytosis.

The initial high activity of the microsomes and the subsequent appearance of most of the antigen in the mitochondria suggest that the antigen is at first adsorbed to the large surface of the small granules and that it later passes into the larger granules. Another possibility to be considered is the incorporation of the microsomes with their load of antigen into the large granules.

The persistence of the antigen in the large granules is particularly interesting because the mitochondria have been considered as self-reproducing units, i.e. as sites of protein synthesis (4). Possibly they are also the sites of antibody synthesis. This is in accordance with our view that antibody synthesis is essentially protein synthesis modified by the presence of antigen (5).

The distinct activity of the nuclei (table 1) is in agreement with recent findings of Coons and his co-workers (6). It is not clear from our experiments whether the presence of antigen in the nuclei is significant, and whether the nuclei are to be considered as antibody producing sites. The fact that immunity is never transmitted to de-

[1] Supported by research grants from the National Institutes of Health, U. S. Public Health Service, and from the U. S. Atomic Energy Commission, Division of Biology and Medicine.

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scendants makes a role of the nuclei in antibody synthesis improbable.

Labelled antigens were also used in an attempt to elucidate the mechanism of the combination of antigen with antibody. Rabbits were immunized by injections of azoproteins and the immune sera were precipitated by the homologous antigens. The precipitates were washed with small amounts of 0.9 per cent saline solution, then suspended in large volumes (35 ml.) of saline solution for the determination of their solubility at different temperatures. After centrifugation it was found that the supernatant solution contained mainly antibody, while only traces of antigen were dissolved. Evidently, the precipitated antigen-antibody complex dissociates according to the equation $GB_n \rightarrow GB_{n-1} + B$, where G = antigen and B = antibody. The dissociation constants found were of the order $K = 10^{-6}$ to 10^{-7}. The thermodynamical constants for combination of antigen and antibody, calculated from the values of 1/K at two temperatures, are recorded in table 2.

TABLE 1. INTRAVENOUS INJECTION OF IODO-OVALBUMIN INTO RABBITS

AMOUNT OF ANTIGEN INJECTED (MG.)	38	80	70	43	65	65	86
TIME (IN HOURS AFTER INJECTION)	0.083	1.25	9.25	91	212	380	696
Blood	65.6	13.9	0.947	0.062	0.025	0.014	
Spleen	0.31	0.38	0.050	0.0013	0.0009	0.0011	
Lungs	3.76	0.30	0.042	0.0072	0.0058	0.0049	0.0026
Kidneys	2.07	1.24	0.157	0.024	0.065	0.013	0.0063
Liver	16.1	16.9	2.52	0.151	0.088	0.048	0.018
Liver fractions							
Nuclei	0.81	0.78	0.93	1.49	1.57	1.77	
Mitochondria	1.74	3.58	3.03	2.58	3.31	3.90	
Microsomes	1.95	1.02	0.69	0.29	0.25	0.21	
Supernatant	0.53	0.30	0.34	0.44	0.42	0.39	

The figures pertaining to organs indicate percentage of the injected activity; those pertaining to liver fractions indicate relative activities, when the activity of the liver homogenate in counts per minute per milligram is set equal to 1.0.

TABLE 2. THERMODYNAMICAL CONSTANTS FOR THE COMBINATION OF ANTIGEN AND ANTIBODY

ANTIGEN Type	Amount	IMMUNE SERUM	t_1	ΔF_1	t_2	ΔF_2	ΔH	ΔS
	mg.	*ml.*	°C.		°C.			
Arsanil-beef serum globulin	2.0	6.5	5	−8.0	25	−8.4	−2.0	21
	4.0	6.5	5	−8.0	25	−9.3	−2.1	21
Sulfanilovalbumin	2.0	11.0	4	−8.5	29	−9.0	−2.7	21
	4.0	11.0	4	−8.7	29	−9.2	−3.0	21

ΔF and ΔH in kcal/mole, ΔS in kcal/mole/degree.

While the free energy change (ΔF) and the heat (ΔH) of combination are negative, the entropy change is positive. The positive value of ΔS is unexpected because the combination of molecules is generally accompanied by a decrease in entropy. The increase in entropy indicates that the combination of antigen with antibody is accompanied by a release of water molecules from the combining sites of the macromolecules.

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ANTIBODY FORMATION IN SURVIVING TISSUES[1]

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AN ATTEMPT has been made to develop a technique for determining the capacity of various tissues for synthesizing antibodies *in vitro*. This technique is designed to avoid the difficulty of determining whether antibody present in a tissue has been synthesized by that tissue or whether it has been transported to the tissue from its origin at another site.

By immunizing animals and sacrificing them at a time when their serum antibody titer is high and, presumably, when antibody is being synthesized actively, it is possible to obtain surviving tissues which are still capable of forming antibody. Incubation of a tissue, which is synthesizing antibody, with an isotopically labeled amino acid permits the utilization of the amino acid for the synthesis of new antibody protein. The capacity of a tissue for forming antibody may be indicated therefore by the presence of isotope in the antibody which is obtained by precipitation with its specific antigen.

METHOD

Young adult rabbits were injected intravenously with a suspension of formalin-killed type VIII pneumococci. Approximately 4 billion organisms were injected 3 or 4 times per week, and the animals were killed by exsanguination after a minimum of 6 weeks of such immunization. Tissues were removed and slices of 0.5-mm. thickness were prepared. Approximately 10 to 15 gm. of wet tissue (less in the case of spleen control) were placed in 50 cc. of medium of the following composition (mM/l.): Na, 142.5; K, 10; Ca, 2.5; Cl, 117.5; HCO_3, 40. About 7000 U of penicillin and 10 mg. of C^{14} carboxyl-labeled glycine ($CH_2NH_2C^{14}OOH$) were added to each flask. A stream of 95 per cent O_2 and 5 per cent CO_2 was passed through the mixtures for 1 to 3 minutes; the flasks were then stoppered, and the preparations were incubated with agitation for 4 hours at 37° C. After incubation, the medium was separated from the tissue slices by centrifugation at high speed. The clear medium was concentrated 5- to 10-fold by negative pressure dialysis against buffered saline. Antibody in the medium was precipitated by the addition of an appropriate amount of pneumococcus type VIII polysaccharide. The specific precipitate was washed with cold saline three or four times and determinations of the radioactivity of the specific precipitate were carried out with a Q-gas counter. All samples were counted for a minimum of 30 min-

TABLE 1. C^{14} ACTIVITY OF PNEUMOCOCCUS VIII SPECIFIC PRECIPITATE

TISSUE	WET WEIGHT OF TOTAL TISSUE INCUBATED	TOTAL AMOUNT OF $CH_2NH_2C^{14}OOH$[1] ADDED	TOTAL ANTIBODY N	C^{14} ACTIVITY[2] OF SPECIFIC PRECIPITATE
	gm.	*mg.*	*mg.*	*counts/min.*
Liver				
Control[3]	90	90	25.0	2
Experimental	180	120	4.6	93
Kidney				
Control[3]	20	20	6.4	8
Experimental	30	30	1.9	7
Spleen				
Control[3]	4	10	4.4	0
Experimental	10	10	0.25	630[4]

[1] C^{14} activity of the glycine is 1.25×10^5 counts per minute per standard dish of glycine of infinite thickness.

[2] Expressed as C^{14} activity per standard dish of specific precipitate of infinite thickness.

[3] Tissues from a non-immunized animal with pneumococcus VIII antiserum added to the medium.

[4] This value is approximate because of the correction of the actual counts (126) to counts at infinite thickness.

[1] This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

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utes; the radioactivity is expressed as counts per minute above background.

The possibility that the radioactivity of the specific precipitate might be due to adsorption or co-precipitation of radioactive glycine or of any of its derivatives was considered. To some extent, dialysis reduced the possibility of adsorption, but to test the second possibility the following control experiment was done. Tissues from a normal non-immunized rabbit were removed and prepared in the same manner as in the previous experiment; in addition, however, serum from a rabbit which had been immunized to pneumococcus type VIII was added to the medium before incubation. The preparations were incubated, centrifuged, dialyzed, and the antibody precipitated with type VIII polysaccharide.

RESULTS

Results of these experiments are presented in table 1. Work is now in progress to confirm and to extend these preliminary findings.

The results indicate that the spleen and liver are capable of synthesizing antibody *in vitro*. Synthesis of antibody by splenic tissue has been described by Parker (1), Fagraeus (2), and Roberts and White (3). Thus far we have been unable to demonstrate such synthesis in kidney tissue. The absence of significant radioactivity in the control indicates that non-specific adsorption or co-precipitation of isotopically labeled products does not account for the isotope activity observed in the specific precipitates of the experimental preparations. However, control preparations differ from the experimental preparations in this respect: in the latter some antibody is presumably intracellular and may be exposed to conditions different from those of the control preparations in which the added antibody may remain extracellular. Whether this consideration is significant cannot be determined at the present time.

DISCUSSION

These findings indicate that this technique affords a means of demonstrating the ability of tissues to form antibodies *in vitro*. By studying the ability of individual tissues and of smaller structural units to synthesize antibody, it may be possible to delineate more clearly the sites of antibody formation.

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BEHAVIOR AND PERSISTENCE OF AZOPROTEIN TRACER ANTIGENS IN MICE[1]

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TO DETERMINE the sites from which the initial stimuli to antibody formation arise the fate of certain tracer antigens is under study. A recent paper (1) describes the preparation of some deeply blue, antigenic azoproteins, and the methods for ridding these preparations of unlinked or free dye. Following intravenous injections of these substances into mice some sort of blue material appears in various tissues and remains in them for weeks. However, the visible presence of color in tissues, like the demonstration of radioactivity following injections of radioactive tracer antigens, is no proof that intact antigen is localized in the cells. The dye may be split off rapidly while the dye protein is still circulating in the blood, so that free dye enters the cells, or, the dye may be split off from the protein after it enters the cells, and there be retained. Further, the protein portion of the azoprotein may be broken down sufficiently to lose its antigenicity, either while in the blood or soon after being taken up by the tissues.

To eliminate the first of these possibilities an extremely diffusible dye, echt-saure-blau, has been diazotized and coupled to either bovine γ-globulin (1) or to human albumin.

This dye is not degraded in the bodies of mice injected with it; instead it is so diffusible that it is rapidly eliminated from the body in 2 or 3 hours without being stored intracellularly as granules, or in vacuoles. The dye does not, by itself, form combinations with proteins. Clearly, if such a dye, coupled to a protein and injected into mice, should become split off from its protein while in the blood stream, no blue matter would appear in the cells; if split off after its entrance into the cells, it would be rapidly eliminated from the body.

By contrast, injections of the two azoproteins, formed from this dye, unlike injections of the dye itself lead to the progressive accumulation and storage of blue granules in many organs, especially in the Kupffer cells of the liver, reticuloendothelial cells throughout the body, and in the kidney tubules.[2] Consequently some sort of linked dye protein, not necessarily intact antigen, is taken up and stored. Is this material the original antigen?

STORED BLUE MATERIAL IS RETAINED ANTIGEN

Complement-fixation tests readily answered this question when carried out upon liver emulsions and blood from mice injected intravenously with the azoglobulin. Soon after the injections the deeply colored blood yielded strong reactions for antigen, the lightly colored liver tissue contained some too, but in smaller amounts, a finding that might be attributed to liver blood. However, as time passed, both color and antigen titer decreased in the blood and increased in the liver, until, in two or three days the organ held far more of both than the blood (1). The blue matter accumulating in the liver seemed to be active antigen. After a fortnight only faint traces of antigen remained in the blood, but, by contrast, the blue material, whether antigen or not, was retained much longer in the livers and mesenteric lymph nodes, indeed all the intracellular colored matter did not disappear from these organs for 85 to 120 days.

The finding suggested that antigens may persist in very small amounts, within certain cells, longer than is generally supposed, thereby stimulating prolonged antibody formation. To investigate the matter it seemed wise to study the

[1] The present note summarizes briefly some findings come upon in this laboratory during the course of work carried out with certain azoprotein tracer antigens. Since some of the work has already been published and the remainder is in the process of publication *in extenso* elsewhere, descriptions of the physiological reactions here mentioned and the presentation of data will be omitted.

[2] Very recent work has shown species differences in the distribution of these azoproteins. In rabbits, for example, the kidney tubules take up far more of the blue material than the livers or mesenteric nodes, although both of the latter contain much. This is the reverse of the state of affairs in the mouse.

persistence of a natural antigen, preferably the protein from which the artificial tracer was derived; because, should the natural antigen be destroyed rapidly in the body, the fate of the artificial tracer would be of little interest. Accordingly an attempt was made to determine the full span of persistence of even the faintest traces of bovine γ-globulin detectable in the blood and certain organs of mice, intravenously injected with it. Since it seemed most unlikely that the minute amounts of antigen to be detected could be successfully extracted from organs for complement-fixation tests, resort was had to a more delicate method for the pursuit of the problem.

EXTREMELY SENSITIVE TEST FOR DETECTION OF TRACES OF ANTIGEN WITHIN TISSUES AND BODY FLUIDS

An earlier paper (2) has described a technique by which extraordinary spasms and other specific vascular reactions in the blood vessels of the ears of anaphylactically sensitive mice are rendered visible under the microscope when the animals are challenged with an intravenous, shocking dose of antigen. These reactions occur even in mice too weakly sensitive to show any of the other signs of anaphylaxis. It now appears that similar ear vascular reactions, hereafter termed EVR, occur in mice injected with various antigens and challenged later with appropriate antisera (reversed passive anaphylaxis). The EVR are so sensitive that one can inject as little as 0.5 to 0.1 μg. of bovine γ-globulin, as antigen, into the peritoneal cavities of normal mice of the Rockefeller Institute strain[3] and detect the absorption of some of it by a subsequent injection of very strong rabbit anti-bovine γ-globulin serum. A description of these reactions and their differentiation from certain physiological reactions which occur from time to time in the ears of intravenously injected, normal mice will be presented fully elsewhere (3), in a paper now in press, since the matter is a complicated one, and it cannot be adequately presented in a brief summary.

The extreme sensitivity of the EVR made it seem likely that mice could be used to advantage, as test animals, to detect the presence of truly minute traces of antigen persisting in the tissues or body fluids of animals previously injected with it.

[3] Different strains of mice have now been found to vary enormously in their sensitivity to reversed passive anaphylaxis. The strain used for this work is especially sensitive.

TESTS FOR PRESENCE OF ANTIGEN PERSISTING IN BLOOD OR TISSUES

Normal mice were injected intravenously with 5 mg. of bovine γ-globulin (0.1 ml. of a 5% solution) per 30 gm. of body weight. At various intervals samples of blood and ground liver, or mesenteric nodes, from these animals (donors) were injected intraperitoneally into normal mice (recipients). Two days later, before antibody formation could take place, the recipients were intravenously injected with strong, rabbit anti-bovine γ-globulin serum. The occurrence of the specific type of EVR indicated the absorption, from the peritoneum, of antigen present in the materials transferred from the donor mice. Suitable controls were employed at all times.

PERSISTENCE OF ANTIGEN

In two series of experiments recipient mice showed specific EVR after transfer of blood from donors injected with the antigen 56 days previously. All reactions after longer periods were negative. The transfer of liver tissue, up to and including 101 days after injecting the donors with antigen, sensitized the recipients sufficiently to yield positive, specific EVR upon the injection of the antiserum. There was therefore a striking similarity in the period of persistence of the bovine γ-globulin, as antigen, in the livers of these donors (101 days) and that of the blue material, whether antigenic or not, in the livers of the other mice injected with the azoglobulin (85–120 days), as mentioned above. The finding called for a direct experiment to find out: Does the transfer of tissues from donor mice, injected with an azoglobulin, to normal recipients lead to the production of positive EVR as long as the transferred tissues show the visible presence of colored material of some sort in them? If so, it is most likely that the colored material is still the antigen.

AN AZOPROTEIN INJECTED INTO MICE SEEMS TO RETAIN ITS ANTIGENICITY AS LONG AS COLOR REMAINS VISIBLE IN TISSUES

A direct test was accordingly made. Since the 101-day period, over which positive EVR had been obtained by the transfer of liver tissue from mice injected with plain bovine γ-globulin, was so much like the 85- to 120-day persistence of color in the livers of other mice injected with

the azoglobulin, presumptive evidence seemed to be already at hand to indicate that the colored matter was still antigenic, at least as far as the azoglobulin was concerned. It seemed wise therefore to employ a different protein for the direct test, and, by so doing, to answer not only the question asked above but also two other related questions: Does an azoprotein persist, as an antigen, for the same length of time as the protein from which it is derived, and, do different proteins persist, as antigens, for unequal periods of time?

To answer these questions, a new azoprotein, prepared by coupling to human albumin the same dye that had previously been linked to bovine γ-globulin, was injected into scores of mice, as donors. At weekly intervals thereafter the presence of antigen, persisting in the blood and certain tissues in the donors, was sought by the detection tests mentioned above. As time passed, the blue material, stored in the tissues of these mice injected with the azoalbumin, disappeared far more rapidly than had the colored matter after like injections of the azoglobulin. At the 23rd day the recipients, which got transferred blood from these donors, no longer showed positive, specific EVR. At the 36th day transfer tests made with ground liver tissue, which contained only faint traces of blue material, yielded very weak, positive EVR in the recipients. The transfer of ground mesenteric nodes, which contained more persisting blue material than the livers, gave somewhat stronger detection tests (EVR) for antigen in other recipients.

Highly significant were two series of tests performed 42 and 43 days after injecting the azoalbumin into the donor mice. The livers of these animals showed no remaining blue when examined grossly, or when magnified at low powers (10 or 20 times), under a dissecting microscope. By contrast, the mesenteric lymph nodes held faint traces of blue. The recipients that got transferred liver tissue from these donors yielded negative EVR, whereas transfer of the faintly colored nodal tissue gave positive EVR. Clearly, as long as the blue material remained visible in the tissues, the detection tests for antigen remained positive.

PLAIN ALBUMIN AND DYE-AZOALBUMIN YIELD POSITIVE DETECTION TESTS FOR SIMILAR PERIODS

Scores of other donor mice were intravenously injected with plain human albumin, instead of the azoalbumin, and transfer tests from these donors were carried out in the same manner. The EVR in the recipient mice became negative at the same time intervals as in the experiments with donor mice injected with the coupled azoalbumin; that is to say, the protein and the azoprotein derived from it, yielded positive detection tests for approximately the same length of time.

It is to be stressed that control tests, too complex to describe here, showed that the appearance of the EVR in all this work could not be attributed to traces of antigen in the antisera reacting with antibody transferred to the recipients from the donors.

WIDELY DIFFERENT PROTEINS YIELD ANTIGEN DETECTION TESTS FOR UNEQUAL PERIODS

The experiment just described answered the last of the questions posed above. Both the plain human albumin and the coupled dye-azoalbumin yielded positive detection tests on the 42nd day, after injecting the donors, but not thereafter, while the bovine γ-globulin yielded positive EVR for 101 days.

BEHAVIOR OF COLORED RESIDUES INDICATES AN UNCHANGED STRUCTURE

It is of much interest that, following the injection of plain globulin into donor mice, the detection tests for that antigen in their tissues remained positive two and one-half times as long as tests for the detection of plain albumin in the tissues of donors similarly injected with that protein. In complete accordance with this, the persistence of colored matter in the livers of mice injected with the azoglobulin and the azoalbumin, respectively, differed in the same manner. Yet the same dye was coupled to both proteins.

Had the presence of color in these tissues, long after injection, been due to dye, split off from the protein to which it had originally been coupled, and either free in the cells or bound to cellular proteins, then its persistence should have been the same in all the tests. But this was not the case. In mice injected with azoglobulin color persisted in the liver for 85 to 120 days; in the animals injected with azoalbumin color could be found in that organ for less than 42 days, and for only a few more days in the mesenteric lymph nodes. These differences are similar to the periods during which positive detection tests can be elicited after injecting the respective azo-

proteins. From all this it would seem to follow that the color behaved as though still coupled to its respective protein.

COMMENT

Since the blue tracer antigens seem to retain their antigenicity as long as blue color can be seen in the tissues they should serve not only to indicate the sites from which the initial stimuli to antibody formation must emanate, when tissues are examined a few days after injecting the animals, but they should show the whereabouts of antigen during prolonged antibody formation.

Little has been said of the distribution of these azoantigens, because a previous paper has fully described the state of affairs during the first few days after injection (1) when the blue material, taken up by the cells, has been definitely shown to be still antigenic. It has seemed best to delay an exhaustive study of the later distribution of the blue matter seen in the cells, long after injecting mice with the azoproteins, until the work here described could indicate with more certainty whether or not the blue material is still the antigen itself, and until more could be learned about the span of persistence of some natural antigens. Such a study will be undertaken shortly.

In conclusion it is necessary to note only that the presence of intravenously injected azoprotein antigens within certain cells of the reticuloendothelial system, long after detectible amounts have disappeared from the blood, is highly significant for hypotheses of antibody formation, for among the various reticular cells in lymph nodes and elsewhere, that take up and store these colored antigens, are some of those that may give rise to plasma cells, lymphocytes, and other cells now suspected of carrying antibody. What happens between the storage of antigen by these reticular cells and the appearance of antibody in their daughter cells is yet to be learned.

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RADIOLABELLED ANTITISSUE ANTIBODIES

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THERE have been many studies made in the field of antitissue antibodies especially in connection with certain diseases which appear to have an etiology of autoimmunization in which the mechanism of the disease process is a production by the individual of antibodies against his own tissue and a subsequent reaction of these antibodies with the tissues. Some diseases which appear to be of this category are glomerulonephritis, multiple sclerosis, rheumatic heart disease, certain anemias, periarteritis nodosa, lupus erythrematosis etc. Also many attempts have been made since the turn of the century to use antisera prepared against malignant tissue, therapeutically, in the treatment of cancer. It seems reasonable that if an antibody which can produce a physiological effect is present in an antiserum it must first interact with that tissue in order to produce such an effect. Thus, progress would be made if it could be demonstrated that there are antibodies in antitissue sera which actually go to the tissue in question. Further, it is not impossible that, if antibodies can be found which go specifically to a tumor tissue, they can be made to carry physiologically active amounts of radioactivity to the tissue.

We have been able to trace the *in vivo* localization of the injected anti-kidney and anti-lung antibodies by labeling them with radioactive iodine (1, 2) or radioactive sulfur (3) and following the localization of the radioactivity. The radioactive labels can be easily attached to antibody molecules without destroying antibody activity (1, 3, 4) and the antibody can be followed very simply by following the attached radioactivity by a counting or radioautographic technic.

Actually, the fraction of the total protein in a serum which is localizing antitissue antibody is very small. Only a small fraction of the total radioactivity in a labelled serum is attached to localizing antibody since the label is distributed over all of the proteins present. Therefore suitable controls of normal serum or antiserum specific to a heterologous antigen were run. Positive antitissue localizing activity was shown by a significantly greater localization in the tissue from the antitissue serum than from the controls. The background radioactivity was reduced by labeling only the globulin fractions of the antiserum.

It has been possible by the use of such labelled antisera to show that antibodies present in antisera prepared against kidney tissue localize to a high concentration in the kidney with some localization in the liver, and antibodies in sera prepared against lung tissue localize primarily in the lung and kidney. In these experiments the antisera were prepared by injecting rabbits with rat lung or kidney tissue. The globulin fractions were obtained by fractionation with alcohol or ammonium sulfate and were labeled by iodinating with iodine containing I^{131} or by coupling with diazotized *p*-sulfanilic acid containing S^{35}.

The duration of the radioactivity which localized in the kidneys indicated that the half life of the localizing antibody in the kidney is of the order of 20 days (5). Thus it appears that the antibody localizing in the kidney is fixed in such a manner that it cannot be metabolized by the cells. Moreover the radioantibody is localized very rapidly, within a single passage through the kidney (6), and the localization takes place primarily in the glomerular tuft (3).

Because of the rapidity of the localization, the localization must take place on some structure on which there is very extensive and complete contact between the blood stream and antigen responsible for the localization. Such contact probably obtains only between the blood and the vascular bed of the organ showing specific localization. Moreover the localization probably takes place on the surface of the cells responsible for this localization. Further evidence supporting this assumption is that the antigen in kidney tissue which causes the localization is insoluble in saline.

We have shown that the antisera prepared against whole kidney tissue contain antibodies which will react with soluble components of kidney tissue and antibodies which can react with insoluble components (7). However it is only

those which react with insoluble components which are responsible for the localization effect. The nonlocalizing antibodies are probably formed against the soluble components of the kidney which are apparently intracellular and the corresponding antibody molecules are nonlocalizing since they apparently do not pass through cell membranes rapidly enough to combine to any large extent with the internal antigens. The parallelism between the properties of the localizing antibody and the nephrotoxic factor as reported by others indicates the identity of these antibodies.

Recently we found that it is possible to elute the antibody which has localized and that this purified antibody can go specifically to the tissue from which it was prepared (8). Thus anti-rat-kidney antibodies eluted from the kidneys of rats receiving radioiodinated anti-rat-kidney serum goes to the kidneys of recipient rats to the extent of 10 per cent whereas only about one-half per cent of the original radioactivity localized in the kidney of the donor rat. However there are also antibodies in anti-rat-kidney serum which localize somewhat in the liver and lung. When the antibodies are eluted from these organs they have the property of returning to the kidney and also to the organ of the original localization. Thus, radioactivity originally localized in the liver, after elution, goes back to the liver and kidney, while material originally localized in the lung goes back to the lung and kidney. All of these antibodies were originally prepared against the kidney. Similar results were obtained with specific purification of antilung antibodies. The localization in the various tissues reflects the heterogeneity of the antibodies.

That the different organs tested can fractionate the antiserum and preferentially remove certain antibody components indicates differences in the vascular beds of these organs which can be observed with the techniques described here.

In those perivascular diseases which appear to have an etiology of autoimmunization it may well be that the damaging antibody is antibody to the vascular bed of the tissue affected and this damaging antibody localizes as rapidly as it is formed. The specificity of the vascular bed would then determine which organ is affected.

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AMERICAN ASSOCIATION OF IMMUNOLOGISTS

Symposium on Hemagglutination by Viruses Which Do Not Specifically Elute[1]

Chairman: GEORGE K. HIRST

IN VITRO REACTIONS OF PNEUMONIA VIRUS OF MICE (PVM)

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PNEUMONIA virus of mice (PVM), a latent infectious agent, may produce extensive viral pneumonia and often death in its natural host, the mouse (1). The study of the pneumonia produced by this agent as an experimental model for the study of a viral respiratory disease (2–4) has been materially aided by the demonstration by Mills and Dochez (5) that this pneumotropic virus, under proper conditions, produces hemagglutination of mouse red blood cells. In addition, this phenomenon has permitted detailed investigation of the reactions between PVM and host cells (2, 6–8). This paper will be concerned with these reactions and the essential factors involved.

Tissue fluid expressed from whole (5) or sliced (9) PVM-infected lungs produces agglutination of mouse RBC, whereas suspensions of ground infected lungs do not clump erythrocytes. However, if such lung suspensions are heated at temperatures from 65° to 80°C. for proper periods of time (2, 5), hemagglutination of added mouse RBC results. Addition of fresh mouse lung suspension again inhibits the agglutination reaction. This phenomenon can be repeated indefinitely without altering the virus. When heated PVM and mouse RBC are mixed and aliquots removed at intervals, it is readily shown (2) that not only does virus combine with the erythrocytes, but also that spontaneous dissociation does not occur. This is at variance with the influenza-mumps-NDV group of viruses which do release themselves from the bound state (10). These facts suggest that, although virus may be in a free state in the intact lung, disruption of the cells results in combination of PVM with a heat labile cell component. The fact that viral preparations which do not produce agglutination of mouse RBC are not filterable through Coors no. 3 filters, and are readily sedimented by high speed centrifugation, whereas preparations which hemagglutinate mouse RBC are readily filtered but cannot be easily sedimented in high gravitational fields (6, 9) is evidence that PVM actually combines with tissue particles. PVM in the combined state is much larger than the free viral particle, of the order of 140 mμ as compared with 40 mμ (9). That the particles which react with mouse RBC are indeed PVM particles has been established (2).

Dissociation of PVM from tissue component can be accomplished not only by heat or high pH (2, 6), but also, as indicated in table 1, by reducing the electrolyte concentration of the suspension (8). Thus, preparation of PVM-infected mouse lung suspension in distilled water or 0.25 M dextrose or sucrose permits direct hemagglutination of mouse RBC, and the free virus is fully infectious. The addition of NaCl to 0.15 M concentration results in combination of virus and cellular material which indicates that the reduced electrolyte concentration does not alter the capacity of either the virus or lung particles to combine. Furthermore, repeated dissociation and association of the PVM-cell component complex can be accomplished. A similar dependence upon the electrolyte concentration and pH is observed for the PVM-mouse RBC complex (8). The ionic requirements are not specific, either mono- or bivalent cations can be employed without influencing the capacity of lung particles or mouse RBC to bind virus. When free infectious virus is employed to demonstrate PVM-RBC combination, the virus remains infectious when converted again to the free state. Furthermore, the RBC are unaltered by combination with virus, and subsequently show undimin-

[1] May, 2, 1951, Cleveland, Ohio.

ished capacity to recombine with virus. Sequential cycles of combination and dissociation of PVM and mouse RBC can be repeated at will by appropriate regulation of the electrolyte concentration and pH of the mixture (8). In contrast, the influenza-mumps-NDV group of viruses irreversibly alter the cell surface of erythrocytes with which they combine, and subsequent combination of virus with the same cells does not occur (10).

Early investigations (2, 7) suggested that PVM combined only with a component in those lung tissues which are susceptible to infection with this agent. Subsequent studies (11), employing more sensitive methods, indicate that, although combination is greatest with particles from cells which are susceptible to infection, PVM can nevertheless combine with a number of mammalian tissues in which this virus does not multiply.

TABLE 1. PREPARATIONS WHICH YIELD PNEUMONIA VIRUS OF MICE IN VARIOUS STATES

STATE	PREPARATION
1. Free infectious	Infected lung—squeeze Infected lung—slice—squeeze Infected lung suspension with <0.01 M electrolyte
2. Free noninfectious	70°C/30 minutes pH 11.0
3. Combined infectious	Infected lung suspension with >0.1 M electrolyte
4. Combined noninfectious	'2' + fresh lung particles

TABLE 2. PROPERTIES OF PNEUMONIA VIRUS OF MICE (PVM) IN RELATION TO ITS STATES

STATE OF VIRUS	PROPERTIES						
	Infectivity mice	Hemagglutination		Combination with lung particles	Specific complement fixation	Specific antigenicity	Size mμ
		Unheated	After 70°C.				
Free infectious	+	+	+	+	+	+	40
Free noninfectious	0	+	+	+	+	+	40
Combined infectious	+	0	+	0	0	+	140
Combined noninfectious	0	0	+	0	0	+	140

In not a single instance, however, has it been possible to demonstrate the association of PVM with tissue of the chick embryo, an animal completely resistant to infection with this virus (2, 7). Combination between PVM and various adsorbents is likewise demonstrable (11). The combination of PVM with tissue particles or RBC appears to be more dependent upon the ionic composition of the milieu than is the formation of similar complexes by the influenza viruses (cf. table 3) (8).

It is now clear that PVM may be obtained in four separate and distinct states: *1*) free infectious; *2*) free noninfectious; *3*) combined infectious; and *4*) combined noninfectious (6). The methods by which virus in each state may be obtained are summarized in table 1. Experimental data suggest that for the most part PVM exists in the infected mouse lung in the 'free infectious' state, but whether the other states exist naturally in the infected host has not been determined. Certain data (12) suggest that an infected mouse lung suspension which is able to produce a progressive infection in the mouse contains both infectious and noninfectious viral particles, both of which produce agglutination of mouse RBC. Table 2 summarizes some of the biologic and physical properties of the viral particles in each state. Although the mechanism by which PVM in the combined infectious state produced infection in the mouse lung was initially puzzling, it has been demonstrated that the lung possesses an enzyme which liberates infectious virus from the combined state (7). The free infectious virus may then combine with susceptible host cells, invade these cells, and multiply to form new particles which then lead to pneumonia (3).

It is evident that the reactions between PVM and tissue particles or mouse RBC are different from those of the influenza-mumps-NDV viruses with comparable cells. Some of these reactions are summarized and compared in table 3. It will be noted that neither inactivated animal nor human sera contains a substance, other than antibody, which reacts with PVM to inhibit combination with mouse RBC, whereas the pres-

ence of such nonspecific inhibitors of hemagglutination by influenza viruses has been widely observed (13). The other reactions likewise clearly denote the marked differences in biological activities of these viruses. It appears that these viruses have dissimilar host cell requirements for multiplication: *1*) the influenza viruses do not interfere with the multiplication of PVM in the mouse lung, nor does PVM preclude the multiplication of the influenza viruses (14); and *2*) mumps virus interferes with the multiplication of PVM but not with influenza viruses in the mouse lung (15).

TABLE 3. COMPARISON OF CERTAIN REACTIONS OF PNEUMONIA VIRUS OF MICE (PVM) AND INFLUENZA VIRUSES

REACTION	PVM	INFLUENZA
Reaction with RBC	Mouse; hamster	Chick +
Combination RBC and tissue particles	+	+
Spontaneous dissociation	0	+
Virus alteration of 'binder'	0	+
Chemical nature of 'binder'	Protein	Mucoprotein
Electrolyte requirement:		
Combination	>0.10 M	>0.0015 M
Dissociation	<0.01 M	
Enzymatic inactivation of 'binder'	Trypsin	RDE (*V. cholerae*)
Enzymatic inactivation of virus	Trypsin; chymotrypsin	0
'Binder' in serum	0	+

DISCUSSION

It has been clearly demonstrated that viruses, animal (8) and bacterial (16), can combine with inorganic materials such as celite and glass, and as such represent artificial complexes. Adsorption of certain viruses to mammalian and fowl RBC may in a sense be equally as artificial a reaction. In certain instances the reaction between virus and erythrocyte has been argued, by analogy, to be similar to or identical with the reaction between virus and the susceptible host cell which supports viral multiplication. That such is not the case with certain neurotropic viruses, GDVII (17), SK-MM group (18), and Japanese B viruses (19), appears probable. By altering the environmental conditions in the test tube, combination of these viruses with the surfaces of certain RBC has been accomplished and agglutination of the erythrocytes occurs. The chemical nature of the surface of the RBC and the chemical constitution of the virus probably dictate the environmental factors required to produce the RBC-virus complex.

Although combination between virus and tissue components or adsorbents may occur in a purely physical sense, it does not follow that certain cell surface constituents are not necessary for combination *in vivo*. Indeed, infection may be limited to those cells which by virtue of their chemical constitution permit cell-virus combination under the environmental conditions which prevail in the host.

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HEMAGGLUTINATION BY VIRUSES AFFECTING THE HUMAN NERVOUS SYSTEM[1]

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DURING the past 18 months, Doctors Edward L. Buescher, Robert M. Chanock and I have succeeded in demonstrating and elucidating some of the properties of the hemagglutinins associated with the viruses of Japanese B (Jap B) encephalitis, St. Louis (SLE) encephalitis, West Nile fever and Russian Spring-Summer (RSSE) encephalitis. While each hemagglutinin possessed distinct properties and presented special problems, certain general principles were found to apply to the entire group.

1) Strains of virus which have had a great many passages in mice (over 100) have failed to yield hemagglutinin by methods which proved successful for strains which have had fewer passages.

2) The electrolyte content and pH of solutions used for extraction of infected mouse brains have determined not only the recovery of demonstrable agglutinin, but also the subsequent stability of that which is recovered. Thus, it has been found necessary *not* to use distilled water, hypotonic buffers, or physiologic salt solution buffered just to the acid side of neutrality, since these extract enough inhibitor to obscure the hemagglutinin. It was also necessary to avoid extraction by means of the Waring blendor. Centrifugation of the 0.9 per cent NaCl extract at 13,000 rpm for 1 hour (approximately 20,000 × *g*) removed particles, which destroyed the Jap B hemagglutinin within about 3 days at 4°C., but left behind another substance capable of destroying the SLE hemagglutinin within about 2 days. A stable SLE hemagglutinin was obtained only after it was found that the substance which destroyed it was most active on the acid side of pH 7.6 and practically inactive at about pH 8.0.

3) The reactions of this whole group of hemagglutinins are markedly influenced by pH, each one having its own optimum narrow zone for reaction with the red cells. However, in the case of the SLE hemagglutinin it was found that this pH zone progressively broadened and shifted to the alkaline side as the preparation 'aged' on storage in the refrigerator.

4) The red cells of choice for the Jap B, SLE and West Nile hemagglutinins are derived from newly hatched chicks, while those from older chicks, chickens, and sheep give lower titers, irregular or negative results. The RSSE hemagglutinin thus far has reacted only with sheep cells. Although these viruses are pathogenic for human beings and rhesus monkeys, they do not hemagglutinate their erythrocytes.

5) The hemagglutinins associated with this group of viruses are all highly unstable at 37°C., and to a varying extent, depending on the virus and the pH, also at room temperature and 2°C. The SLE hemagglutinin is much more unstable than that of Jap B. Thus, it was impossible to obtain reproducible or reliable results in hemagglutination inhibition tests with SLE virus, until it was found that at 2°C. and at pH 6.9, which was optimum for hemagglutination, the diluted hemagglutinin was destroyed within the brief time required to distribute it in a series of tubes, and appropriate modifications in technique were instituted to overcome this difficulty.

6) All normal sera were found capable of inhibiting these hemagglutinins in dilutions as high as 1:2,000 or more, the titer varying with the species, individuals within the species, and, of course, the amount of hemagglutinin in the test. This may be contrasted with the absence of such normal inhibitor for the hemagglutinin of encephalomyocarditis virus (MM, Columbia Sk). The inhibitor in normal serum behaves like soluble receptor substance, and is present in abundance in animals whose erythrocytes have no receptors for these hemagglutinins. The normal inhibitor for the Jap B, SLE and West Nile hemagglutinins is either lipid or lipoprotein, because it proved possible to remove it by extrac-

[1] Aided by a grant from the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board.

tion of serum with chloroform, benzene, ether, etc., or by precipitation of the protein with acetone. Since specific antibody was unaffected by these procedures, it became possible to measure it without interference from the normal inhibitors in the serum.

7) No receptor-destroying enzyme was found to be associated with the Jap B, SLE and West Nile hemagglutinins. However, hemagglutination could be reversed by repeated shaking at room temperature or 37°C., reversal occurring first in the mixtures containing the smallest amount of hemagglutinin. There is evidence that the reaction between hemagglutinin and the receptors on the red cells is of the equilibrium type. Accordingly, as the uncombined hemagglutinin is destroyed at room temperature or 37°C., particularly on the acid side of neutrality, more hemagglutinin is dissociated from the erythrocyte receptors, and hemagglutination is reversed. That the receptors are unaffected in this reaction is evident from the fact that the erythrocytes are readily reagglutinated by the addition of fresh hemagglutinin.

PROPERTIES AND REACTIONS OF THE JAP B HEMAGGLUTININ (1)

Hemagglutination has been demonstrated with each of 9 different strains of this virus, which have had from 4 to about 70 passages in mice. The Nakayama strain, which in its 70th to 80th passage yielded the optimum titers of hemagglutinin, gave completely negative results with material which had had 183 or more passages in mice. Potent preparations with titers as high as 1:10,000, which remain stable for many months in an ordinary refrigerator at about 5°C., can be prepared as follows. The infected mouse brains, washed free of blood, are ground in a mortar with unbuffered 0.9 per cent solution of NaCl to form a 20 per cent suspension. After extraction in the cold for a period of 4 to 12 hours, the suspension is centrifuged in the cold at 13,000 rpm for one hour (about 20,000 × *g*), and the supernatant liquid is stored at 5°C. Centrifugation at only 2,000 rpm for 10 to 20 minutes leaves particles in the supernatant liquid which completely destroy the hemagglutinin in about 4 days at 5°C. and more rapidly at higher temperatures. Removal of these particles not only yields a preparation that is stable in the cold but one whose titer frequently increases 4- to 16-fold during the first week of storage. Hemagglutination occurs only in the pH zone of 6.3 to 7.1 and optimally at pH 6.5 to 6.8. Hemagglutination also occurs when the dilutions are made in unbuffered 0.9 per cent NaCl, but the resulting titers vary markedly with different preparations, with period of storage, and other, as yet, undetermined factors. When appropriately buffered, 0.9 per cent NaCl solution is used for preparation of the dilutions, e.g. M/50 or M/100 phosphate of pH 6.5, titers are enhanced as much as 4- to 100-fold, again varying with the type of preparation, period of storage, erythrocytes used etc. This enhancement does not occur when the dilutions are first prepared in unbuffered 0.9 per cent NaCl, and immediately thereafter adjusted to the optimum pH and buffer concentration prior to the addition of erythrocytes. There is no indication that the unbuffered salt solution is deleterious to the hemagglutinin; it appears rather as if the appropriate buffer and pH causes an unfolding or depolymerization of the hemagglutinin. As a matter of fact after storage in the refrigerator for a month or longer as an ordinary saline extract certain preparations undergo a change whereby dilution in unbuffered 0.9 per cent NaCl gives the maximum high titers without any enhancement by buffers at optimum pH. The absence of hemagglutination immediately outside of the required pH zone is not due to rapid destruction of the hemagglutinin, but rather to failure of combination with the erythrocytes. Hemagglutination cannot be reversed by resuspending the agglutinated, sedimented erythrocytes in solutions having a pH outside the required zone.

A 0.25 per cent suspension of chick cells is used (final concentration of 0.125%) because it yields the highest titers. In solutions properly buffered for optimum dispersion of hemagglutinin (H) and combination with receptor (R), the hemagglutinin titer diminishes in a constant ratio as the concentration of erythrocytes increases. Thus, when the dilutions are made in 0.9 per cent NaCl containing M/50 phosphate at pH 6.5, the titer increases 4-fold as the concentration of erythrocytes, in the range of 2 per cent to 0.25 per cent, is decreased 2-fold. These data, as well as those on reversal of hemagglutination without change in the receptors, suggest that the reaction between H and R is of the equilibrium type which may be expressed as $H_x + R_y \rightleftarrows H_xR_y$, and that the number of R on a single erythrocyte combined with H varies with the concentration of H and the number of erythro-

cytes in the mixture. The lower titers with the more concentrated suspensions of erythrocytes also indicate that a minimum number of R on each cell must be combined with H to result in hemagglutination. It is also worth noting here that, in several simultaneous experiments, the enzymes of *Vibrio cholerae* destroyed the receptors for influenza virus on the chick erythrocytes, but were without effect on the receptors for the Jap B hemagglutinin.

Normal sera from human beings, monkeys, rabbits and mice inhibited hemagglutination, even after the pH was adjusted to the optimum zone required by the hemagglutinin of Jap B virus. The inhibitor in the normal sera reacts with the hemagglutinin in precise multiple proportions giving a straight line relationship with a slope of 1. The inhibitor can reverse the hemagglutination of cells which had combined with small amounts of hemagglutinin (8U or less), and behaves as if it were either the receptor substance of the erythrocytes in soluble form or a substance having an affinity for the same combining groups on the hemagglutinin. The normal inhibitor cannot be removed by dialysis. It is not affected by heating at 65°C., and boiling for 15 minutes does not appreciably alter the inhibitory titer of rabbit and mouse sera but reduces the titer of human serum as much as 75 per cent. The lecithinase of *Clostridium perfringens* toxin, in the presence of $CaCl_2$, does not destroy the inhibitor but abolishes its resistance to boiling. Treatment with periodate or strong proteinases does not destroy the inhibitor. However, extraction with lipid solvents (ether, benzene, ethylene chloride, chloroform) or precipitation with acetone largely or completely removes the inhibitor from rabbit and human serum and only partially from mouse and monkey serum. Chloroform extraction yields the best results, removing at least 95 per cent of the inhibitor but leaving specific antibody in undiminished titer.

The reaction between specific antibody and the Jap B hemagglutinin also gives a straight line relationship but with a slope of <1, thus differing from the direct proportionality obtaining in the reaction with normal inhibitor. Preliminary observations have indicated that the hemagglutination inhibition produced by specific antibody can be utilized for both diagnostic and epidemiologic survey purposes.

The Jap B hemagglutinin is very heat-labile. At pH 6.5 it becomes inactive at 37°C. within 30 minutes, while at pH 7.1 inactivation is only slightly slower. At a room temperature of 24° to 25°C. inactivation proceeds at a regular rate somewhat more slowly. At pH 7.0 to 7.1 the activity persists for months at about 4°C. These data apply to 13,000 rpm supernates from which the inactivating substance or enzyme associated with the particles sedimented at this speed has been removed; before removal of this substance inactivation proceeds more rapidly at all temperatures and is complete a few days at 4°C. The effect of freezing and thawing and of lyophilization differs in uncentrifuged and centrifuged preparations as shown in table 1.

The marked instability of the hemagglutinin is also apparent in the fact that mere bubbling of nitrogen gas through 13,000 rpm centrifuged 20 per cent suspension, diluted 1:16 in 0.01 M borate-saline buffered at pH 6.8 and kept in an ice bath, was enough to inactivate 75 per cent of it in 5 minutes and 96 per cent in 20 minutes, without significant alteration of pH.

TABLE 1. EFFECT OF FREEZING AND THAWING AND LYOPHILIZATION ON JAP B HEMAGGLUTININ

PROCEDURE	20% JAP B MOUSE BRAIN SUSPENSION	
	Uncentrifuged	13,000 rpm supernate
Frozen and thawed once	Active	Inactive
Frozen and thawed 5 ×	Inactive	Inactive
Lyophilization	Inactive	Active

Ultracentrifugation and gradocol membrane filtration experiments indicate that the Jap B hemagglutinin is not smaller than the infectious particle. There is rather a possibility that it may be larger. Centrifugation at 31,440 rpm (average of 58,300 × *g*) which almost completely sediments the infectious particles, also removes all of the hemagglutinin from the supernatant liquid. Although 75 per cent of the hemagglutinin is removed from the supernatant liquid after centrifugation at 21,740 rpm (average of 27,950 × *g*), it is difficult to correlate this with the behavior of the infectious particles, because titration of infectivity is not sufficiently accurate within these limits. However, while the average pore diameter (APD) of the membrane, which just allows minimal amounts of infectious virus

to pass, is 50 mμ, and while almost all of the infective particles pass membranes with an APD of 100 mμ or larger, the hemagglutinin was demonstrable in filtrates from membranes with an APD of 260 mμ or larger, but not from those with an APD of 200 mμ or less.

Of special interest is the effect of certain simple chemical compounds and of certain bacterial products on the Jap B hemagglutinin. Thus, $ZnSO_4$, which had no effect on hemagglutination by influenza virus, was capable of inhibiting the Jap B hemagglutinin in amounts of 0.03 to 1.92 μg. per unit, the more dilute solutions requiring more Zn per unit. It was found that in the concentrations used, the zinc had no effect on the red cells but combined with the hemagglutinin (H) in an equilibrium type of reaction, which may be expressed as:

$$H_x + Zn_y \rightleftarrows H_x\,Zn_y$$

It was possible to reverse this reaction by dilution and by careful removal of Zn from the mixture with H_2S. The experiments with zinc were carried out with collidine or borate buffers. A suitably buffered solution of diethyl, dithio carbamate inhibited the Jap B hemagglutinin in a final concentration of 1:2,000, while 8-hydroxyquinoline and α,α' dipyridyl which also bind heavy metals (although not as well in the presence of proteins) were without effect in 20 times greater concentration. Partially purified preparations derived from cultures of *Clostridium histolyticum* and *Clostridium lentoputrescens*, after boiling for 15 minutes, inhibited the Jap B hemagglutinin in dilutions as high as 1:1,000,000. The effect was on the hemagglutinin and not on the red cells. Further purification of the substances by Dr. A. A. Tytell indicated that the inhibitory activity was associated with certain nucleic acid or protein components which were different from the proteinase in these cultures.

PROPERTIES OF THE ST. LOUIS ENCEPHALITIS VIRUS (SLE) HEMAGGLUTININ

No hemagglutinin could be extracted from mouse brains infected with the Webster no. 3 strain of SLE virus which has had an unknown large number of passages since its isolation in 1933, but was found in several recently recovered strains kindly supplied by Dr. E. H. Lennette. However, the SLE hemagglutinin proved to be much more unstable than the Jap B, and little work could be done with it until the physicochemical factors responsible for its rapid degradation were discovered (2). The hemagglutinin is now prepared by extracting the washed, infected mouse brains with enough M/64 borate buffer of pH 9.0 in 0.9 per cent NaCl to yield a 20 per cent suspension. After centrifugation in the cold at 13,000 rpm for one hour, the supernatant liquid has a pH of 8.0 to 8.1 and hemagglutinin titers as high as 1:2,560.

Although hemagglutination occurs with both sheep and chick erythrocytes, 0.25 per cent suspensions of red cells obtained from newly hatched chicks give the best results. The test is performed at room temperature, although 4°C. is also suitable. With a fresh preparation, a pH of 6.7 is usually optimum and negative results are the rule beyond pH 7.0. As the preparation is aged on storage at 4° C. the pH zone for hemagglutination progressively shifts to the alkaline side and broadens to a point where after a period of about 30 days the reaction occurs best between pH 6.9 and 7.8. At pH 7.0, the SLE hemagglutinin (13,000 rpm supernate + equal volume of phosphate buffer) was destroyed within 5 minutes at 37° C. and within 10 to 20 minutes at 24° C., while at 1° C. it lost 85 per cent of its activity within 5 to 10 minutes. At pH 7.9, on the other hand, the activity persisted in undiminished titer for at least 160 minutes at 24° C., but was still destroyed within 5 to 10 minutes at 37° C. No receptor destroying enzyme was found, but hemagglutination could be reversed by shaking the tubes, the extent of reversal being influenced by pH, temperature and time. Normal sera contain inhibitor which reacts with the SLE hemagglutinin in the same manner as with that of Jap B. Acetone precipitation proved to be superior to chloroform extraction for elimination of this inhibitor, and the precipitated protein contained the specific antibody in undiminished titer. Tests on acute and convalescent sera from 9 patients with St. Louis encephalitis (kindly supplied by Dr. E. H. Lennette), as well as on 50 normal sera, showed that the hemagglutination inhibition test can be used for diagnostic purposes.

HEMAGGLUTININ OF WEST NILE FEVER VIRUS

Although only exploratory work has been done thus far with this virus, a hemagglutinin was obtained in 0.9 per cent NaCl extracts of in-

fected mouse brains which reacted with chick and sheep erythrocytes at a pH of 7.0 to 7.5 (optimum about 7.3) at room temperature. It was thus apparent that the three antigenically related viruses could be differentiated by the pH zones in which they produced hemagglutination (see table 2). Since the pH zone changes markedly for 'aged' SLE hemagglutinin and possibly also for that of West Nile, the data shown in table 2 apply only to relatively fresh preparations.

Little work has been done thus far on the physico-chemical factors which influence the stability and behavior of the West Nile hemagglutinin, but a peculiar phenomenon was observed with reference to the temperature requirements for the reaction between stored hemagglutinin and chick cells (see table 3).

TABLE 2. DIFFERENTIATION OF ANTIGENICALLY RELATED VIRUSES BY PH ZONE OF HEMAGGLUTINATION

VIRUS	HEMAGGLUTINATION AT INDICATED PH		
	6.5	6.7	7.4
Japanese B	++++	+++	0
St. Louis	0 to ±	+++	0
West Nile	0	0	+++

TABLE 3. INFLUENCE OF TEMPERATURE ON HEMAGGLUTINATION OF CHICK ERYTHROCYTES BY STORED OR FROZEN AND THAWED WEST NILE HEMAGGLUTININ

TEMPERATURE OF REAGENTS AT TIME OF MIXING	TEMPERATURE FOR SUBSEQUENT SEDIMENTATION OF ERYTHROCYTES	RESULT
25° C.	25° C.	Negative
25° C.	4° C.	Positive (1:2560)
2°–4° C.	4° C.	Negative

Other tests also indicated that while 25° C. was required for initial combination, 4° C. was required for appearance of hemagglutination. It should be noted, however, that this phenomenon obtained only for *stored* or *frozen and thawed* hemagglutinin and chick cells, since fresh preparations hemagglutinated both chick and sheep cells at 25° C. and stored or frozen and thawed preparations still agglutinated sheep cells at 25° C. However, higher titers were obtained when mixtures prepared at room temperature were allowed to sediment at 4° C. Chloroform extraction removed the normal inhibitor and anti-West Nile sera produced specific inhibition. A common antigen between the Jap B, SLE and West Nile hemagglutinins was also demonstrated by hemagglutination inhibition tests with chloroform-extracted sera.

HEMAGGLUTININ OF RUSSIAN SPRING-SUMMER ENCEPHALITIS (RSSE) VIRUS

Infected mouse brains extracted for several hours in unbuffered 0.9 per cent NaCl, yielded preparations which hemagglutinated sheep erythrocytes (but not chick, human 'O' or rhesus) at pH 7.5 (but not pH 5.5, 6.5 or 8.5) and at room temperature (26° C.) but not at 4° C. These properties differentiate it not only from the hemagglutinins of Jap B, SLE and West Nile viruses but also from that of the encephalomyocarditis-MM-Columbia Sk virus. Preliminary observations suggested the possibility that the RSSE hemagglutinin, unlike those of Jap B, SLE and West Nile, may destroy the receptors on the sheep cells. Normal sera had inhibitory activity which could *not* be removed by extraction with chloroform. While normal guinea pig serum had an inhibitory titer of 1:20, anti-RSSE guinea pig serum had an inhibitory titer of 1:160. The RSSE hemagglutinin is very unstable at pH 7.0 to 7.1, even at 4° C., and much more work remains to be done with it.

HEMAGGLUTININS FOR WESTERN (WEE) AND EASTERN EQUINE ENCEPHALITIS (EEE) AND POLIOMYELITIS VIRUSES

Extraction with unbuffered, 0.9 per cent NaCl has thus far yielded no hemagglutinin for multiple-passaged and recently recovered strains of EEE, WEE, and poliomyelitis viruses in surveys utilizing chick, sheep, rhesus and human type 'O' erythrocytes and diluents buffered at pH 5.5, 6.5, 7.5 and 8.5. One recently recovered WEE strain gave suggestively positive results with chick and sheep cells at pH 5.5 at room temperature, but this observation has not yet been followed up. Extracts of several recently recovered strains of poliomyelitis virus, centrifuged at 2000 rpm, regularly produced hemagglutination of human type 'A' and 'O' cells (but not of chick or sheep cells) at pH 5.4 to 6.2 and at 37° C., but not at room temperature or 4° C. While extracts of normal monkey cords were negative under the

same conditions, the hemagglutination could not be inhibited by specific antiserum and appeared to be due to a substance in the infected tissue with an isoelectric point in this particular pH zone. At the moment it is difficult to indicate the procedures or conditions which may lead to the demonstration of hemagglutinin for the poliomyelitis group of viruses. From what has been presented thus far, however, it is clear that the viruses affecting the human nervous system provide an interesting field for future studies on hemagglutination.

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HEMAGGLUTINATION BY GDVII AND THE COL SK, COL MM, MENGO ENCEPHALOMYELITIS AND ENCEPHALOMYOCARDITIS VIRUSES

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THE AGGLUTINATION of sheep erythrocytes by Col SK and Col MM viruses first was reported in 1947 by Hallauer (1). Olitsky and Yager (2) extended this work by not only confirming the findings of other investigations but by adding the viruses of encephalomyocarditis and Mengo encephalomyelitis. At the same time, Lahelle and Horsfall (3) reported that the GDVII strain of mouse encephalomyelitis virus would agglutinate human O cells in the cold. Later Verlinde and de Baan (4) and Bremer (5) also showed positive sheep cell hemagglutination by Col SK and Col MM viruses. This series of papers (1–5) brought to light for the first time the fact that several neurotropic viruses would cause the agglutination of certain erythrocytes under very exacting requirements of incubation temperatures and that such hemagglutinations were specific in that hemagglutination inhibition (HI) could be demonstrated when immune serum was added in the usual HI test. Interest was thus stimulated to discover other neurotropic viruses capable of agglutinating erythrocytes. Results of the work of Verlinde and de Baan (4) and of Sabin and Buescher (6) have now extended this list of viruses to include Japanese encephalitis, St. Louis encephalitis and others, each of which has its own peculiarities of temperature of incubation, preparation of the antigen, type of cells agglutinated and the pH range. It is believed that the future may reveal other neurotropic viruses to agglutinate erythrocytes provided the exacting conditions for each are discovered.

Lahelle and Horsfall (3) demonstrated that the GDVII strain of mouse encephalomyelitis virus would agglutinate human O red cells at 4° C. but not at 23° C. or 37° C.; that the hemagglutination was inhibited by homologous immune sera and by FA antiserum but not by antiserum against other neurotropic viruses. They failed to demonstrate that FA virus would agglutinate any of the erythrocytes tested under their experimental conditions.

Lahelle and Horsfall (3) observed that where GDVII virus is readily adsorbed by human O erythrocytes at 4° C., it was quickly eluted from them at 37° C. and that this adsorption and elution could be carried on an indefinite number of times. By taking advantage of this property, it was possible to effect about a 10-fold concentration.

Fastier (7) investigated the mechanism of GDVII hemagglutination by heating the antigen to 56° C. for 30 minutes. This reduced the titer considerably but did not influence the adsorption and elution process with human O red cells. Unlike PR8 influenza virus, treatment of human O cells with periodate had very little effect on the elution of GDVII virus. In addition, extracts of human O cells inhibited influenza hemagglutination to a much greater degree than was apparent for the GDVII strain. He concluded that the mechanism of hemagglutination of GDVII and PR8 were not closely related. Fastier (8) later investigated the inhibition of GDVII virus hemagglutination by normal tissue extracts. He concluded that saline extracts of normal mouse organs were, in some varying degrees, capable of inhibiting GDVII virus hemagglutination. Sera deriving from the mouse, rat, guinea pig and rabbit exhibited this property to the largest extent. The inhibitor present in mouse brain and serum reacted similarly to the effects of heat and other agents tested. Studies on the distribution of the inhibitor revealed that fetal mouse brains and adult tissue contained comparable amounts. Fastier showed that the inhibitor could be extracted in saline solution, further, that this material could be concentrated by 50 per cent saturation with ammonium sulfate at pH 7.0. This extract appeared to be incapable of inactivating GDVII virus when used in place of immune serum in neutralization tests.

Before beginning a discussion of the Col MM Col SK, encephalomyocarditis and Mengo en-

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cephalomyelitis viruses, it should be pointed out that Warren and Smadel (9) and Dick (10) have published conclusive evidence that these viruses, probably indigenous in rodents, are identical, such evidence being based on the results of neutralization, complement-fixation and cross-immunity tests. Therefore, in this presentation, these viruses will be referred to as the EMC group as suggested by Warren and Smadel (9).

Each of the viruses of the EMC group will agglutinate sheep cells at 4° C. and will elute if the temperature of incubation is raised above 20° C. Like GDVII hemagglutination such adsorption and elution may be carried on an indefinite number of times and this method may be employed to concentrate the virus. Homologous immune sera will inhibit hemagglutination as well as heterologous immune sera prepared against any of the members of the group. Thus the cross-hemagglutination inhibition (HI) test provides further serologic evidence of their identities.

Gard and Heller (11) working with the Col MM strain of the EMC group have attempted to refine the method employed and standardize a technic of determining HI titers in human sera, a tool to be used to determine the importance of the disease in human beings. A very dilute erythrocyte suspension, 0.05 per cent, was employed to increase sensitivity and the resulting loss of stability was overcome by the addition of an extract of sheep brain in the place of bovine plasma albumen employed by Olitsky and Yager (2). A 10 per cent sheep brain extract clarified by centrifugation at 12,000 *g* was employed as the diluent for the virus propagated in mouse brain. These authors failed to find inhibitors similar to those reported by Sabin and Buescher (6), effective against Col MM hemagglutinin in either infected or normal brain tissue. Hemagglutination was not inhibited by pseudomucinous ovarian cyst fluids which evidenced high inhibitory activity against influenza virus hemagglutinin. In addition, and unlike Japanese encephalitis virus (6), Col MM was shown to be independent of pH range (6.0–9.0). However, ionic composition of the suspending medium was a factor since hypertonic salt solutions suppressed hemagglutination. The presence of positive or negative ions in solution, exerted much greater influence. It was shown that mono- and divalent cations had no obvious effect. On the other hand, multivalent anions caused considerable inhibition about proportional to their charge. Four units of virus would be completely inhibited in the standard HI test by 0.0002 M hexametaphosphate. Evidence was brought forth to show that this action was on the red cell rather than upon the viral particles. For example, if the number of units of virus were increased in the HI test, a corresponding decrease in the HI titer of the serum was observed. On the other hand, the HI titer of hexametaphosphate was independent of the number of units of virus employed to the HI test. In addition sodium hexametaphosphate failed to neutralize the Col MM virus in the standard neutralization test. Gard and Heller (11) propose the use of a standardized inactivated immune rabbit serum which like the test sera is adsorbed in the cold with sheep erythrocytes. The hyperimmune rabbit serum is, in addition, adsorbed with normal mouse brain. An HI test with adequate controls is conducted employing 0.2 ml. of serial dilutions of serum, 4 agglutinating units contained in 0.1 ml. After incubation for half an hour at room temperature, 0.1 ml. of a 0.2 per cent RBC suspension is added and the test is incubated at 4° C. Results are expressed as inhibiting activity in comparison to a standard serum and expressed as the HI index, since the HI titer of a serum is dependent upon the amount and state of the virus employed in the titration. An index of below 1 was classified as negative, 1 to 4 doubtful or weak and values above 8 were considered positive. Results obtained on 384 sera deriving from patients who exhibited CNS symptoms were examined and of this group 73.2 per cent were negative, 14.3 per cent weakly positive and 12.5 per cent positive. Of 39 sera deriving from patients with various diseases other than the involvement of the CNS, no positive reactions were obtained. The 146 sera obtained from blood donors and pregnant women were examined, and 88.4 per cent were negative, 10.9 per cent exhibited weakly positive results and 0.7 per cent were positive. Gard and Heller, however, point out that the positive sera failed to neutralize Col MM virus in the neutralization test and believe that the inhibiting capacity of the sera is not due to the presence of specific antibodies and is therefore, in its present state, not a usable tool for determination of past infection with a virus of the EMC group. On the other hand, since Warren, Smadel and Russ (9) have found antibody to the different members of this group in patients having a mild febrile illness, and since Seligmann and Jungeblut (12) have demonstrated positive neutralizing antibody against the Col SK virus

in normal sera, the question of whether positive reactions obtained by Gard and Heller may not have been due to inapparent or non-recognizable infection. Further work is needed to clear up this point.

Olitsky and Yager (2) attempted to discover other neurotropic viruses that would cause hemagglutination of erythrocytes. Eastern equine encephalitis, Western equine encephalitis, Venezuelan equine encephalitis, Japanese B encephalitis, St. Louis encephalitis, Russian Far East encephalitis, vesicular stomatitis (2 strains), Theiler (FA strain), Theiler (TO strain), poliomyelitis (Lansing strain) poliomyelitis) MEF1 strain), West Nile, rabies, lymphocytic choriomeningitis, herpes simplex and louping ill viruses were all tested for hemagglutinins using sheep, human O, chick, horse, hamster, dog, cat and guinea pig erythrocytes. In no instance was a specific hemagglutination observed that could be inhibited by homologous immune serum. Many nonspecific false or spontaneous hemagglutination reactions did occur, particularly when a mouse brain virus or normal mouse brain suspensions were employed with hamster cells (4). Normal and immune sera was shown to contain an inhibitor (HI) against this hamster hemagglutinin. In addition an agglutinin for dog, cat and guinea pig erythrocytes is contained in normal or virus-infected mouse brain suspensions.

Yager *et al.* (13) employing substantially the same technic used by Olitsky and Yager (2) attempted to extend the hypothesis that hemagglutination might depend upon selection of erythrocytes deriving from a particular species of animal or bird since GDVII will agglutinate only human O cells and the EMC group only sheep erythrocytes. In the course of this investigation, erythrocytes deriving from 25 species were employed using eight viruses as antigens. In no case could a specific hemagglutination be demonstrated.

Bremer (5) demonstrated with reference to hemagglutination of sheep cells by Col SK and Col MM viruses that potassium (C1) enhances agglutination while calcium ($C1_2$) elutes the virus from the cells thus inhibiting agglutination.

Sabin and Buescher (6) have pointed out that in the case of Japanese encephalitis virus hemagglutination not only is the selection of an erythrocyte important as is the ionic state of the medium and also the pH range. In addition, continued passage in mice will cause the loss of the ability of the Japanese encephalitis virus to agglutinate red cells, so that a positive test will result only with viruses in the earliest mouse passages. This finding we have been able to confirm (14).

In conclusion, the EMC group and GDVII strain of Theiler virus will agglutinate erythrocytes, hemagglutination being inhibited by homologous immune serum, but in each of these instances, many variables are encountered and different conditions underlie the reactions. The demonstration of hemagglutination by additional neurotropic viruses is a possibility but the road to this achievement has many barriers as illustrated by the experience of the workers in this field.

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AMERICAN ASSOCIATION OF IMMUNOLOGISTS

Symposium on Growth of Bacterial Virus in the Host Cell[1]

Chairman: MARK ADAMS

MECHANISM OF BACTERIOPHAGE REPRODUCTION[2]

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THE STUDY of phage reproduction centers around the question of how the specific material substrate of the virus is replicated in many copies inside the host cell. A number of approaches have been developed, some of which have been well illustrated by the reports presented by the other participants in this symposium.

The biochemical approach (1–3) analyzes the changes in amounts of gross chemical fractions of the virus-infected system (proteins, RNA, DNA); it studies the origin of virus components by isotopic and by nutritional methods; it follows the fate of the components of the infecting virus and of certain host cell components, particularly enzymes. The results from this approach to date indicate that the new virus is made from building blocks synthesized, partly before but mostly after infection, by the host enzymes, and that in all probability the only *specific* material to be formed in phage-infected bacteria (in infections normally leading to lysis rather than to lysogenicity, that is, to symbiosis between virus and bacteria) is virus material. Phage infection apparently allows the pre-existent enzymes of the host to pile up the building blocks, but redirects the specific organization of these towards its own ends.

We have recently obtained cytochemical data (4) that suggest a possible interpretation of the reorientation of specific syntheses by phage infection. Chromatinic staining of bacteria infected with the same phages used in the biochemical studies reveals an almost immediate disruption of the 'nuclear bodies' of bacteria after infection. This is followed by a filling up of the cell with a finely granular type of Feulgen positive material (chromatin), which we tentatively identify with phage material because of its absence whenever infected bacteria fail to produce active phage, either because of genetic peculiarities of the host or because of pre-treatment of the phage, for example, with proper doses of radiation. Nuclear disruption itself, however, is always present whenever the bacterium is killed by the phage, whether active phage production follows or not. It seems reasonable to suppose that the peculiar 'killing' of bacteria by phage, which suppresses cell reproduction but not cell metabolism, is a direct consequence of the disruption of the nuclear, presumably genetic, apparatus of the bacteria. Furthermore, I wish to suggest that the redirection of the syntheses results from the replacement of the genetic apparatus of the host with that of the virus; under the influence of the new set of genetic determinants brought in by the virus, virus is produced instead of bacterial protoplasm. In particular, the failure to synthesize new bacterial enzymes, as illustrated by the lack of enzymatic adaptation (5), would result from alteration of the corresponding genes. For production of an adaptive enzyme, there is supposedly (6, 7) a need for energy, for building materials (N sources, internal or external), for the specific substrate, and for the corresponding gene; in phage-infected bacteria, the gene would be the missing (or incapacitated) ingredient. According to this hypothesis of 'genetic parasitism', we should consider cases of lysogenicity as examples of 'genetic symbiosis' between phage and bacterium.

It is important to remember that neither these experiments nor any chemical results tell us anything as yet as to *how* the infecting phage acts in impressing specificity on the newly formed

[1] Atlantic City, N. J., April 20, 1950.

[2] Aided by a grant from the American Cancer Society recommended by the Committee on Growth.

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material. Actually, we have as yet no idea as to what chemical fraction of the phage (or of any other biological material) is responsible for specificity, nor as to what role, if any, the nucleic acid or protein fractions of a so-called nucleoprotein may play in replication of specificity.

The 'activity cycle' approach attacks the problem of reproduction at the biological level, by tracing the specific virus material through its ability to reproduce. Reproduction takes place in the latent period between infection and lysis, at the end of which several hundred new active particles may be produced. Premature disruption of infected bacteria (8, 9) reveals, first, an 'eclipse' period, during which *no* active virus is recoverable, followed by the appearance and gradual increase of virus activity in the second half of the latent period. The early eclipse (an apparently general occurrence in virus infections) suggests a reorganization of the virus material into a non-infectious form.[4] What goes on during the eclipse period? Genetic studies throw some light here. As has been described in this symposium, mixed infection with phage particles of two related types differing by at least two characteristics (such differences can originate by mutation) gives rise to recombinant types in rather precise proportions (11, 12). The fact that recombinant types are present with similar frequency in the final yield and among the very first active particles that appear inside the bacterium around the middle of the latent period (9) indicates that, whatever the reorganization that makes recombination possible, it must take place as early as the formation of the first active virus or earlier. The recombinants of reciprocal types formed within individual bacteria are not present in equal or even correlated numbers (9, 13); this suggests that they do not result from reciprocal exchanges of parts among pairs of mature particles. Moreover, the particles of each recombinant type in individual bacteria are distributed at random, without any sign of 'clonal' arrangement. This suggests that recombination occurs at a stage in production of virus after which *that* virus does not act any more as a model for further virus production.

I have recently obtained very different results by studying the distribution of spontaneous phage 'mutants' (rather than recombinants) produced in individual bacteria. This distribution, based on 95 bacteria-liberating mutants of phage T2 out of over 5000 examined, is definitely clonal and quite similar to the one expected if each new mutant gave rise by successive reduplications to a 'clone' of mutant elements. We know that in phage spontaneous mutations occur only during reproduction (14), probably during actual replication of genetic material. The above result suggests then that there is a phase of logarithmic reduplication of phage material, during which mutations occur but recombinants are not yet formed. Recombinants, then, are probably formed after reproduction of phage material but not after the formation of active particles. A possible interpretation is that individual specific units or groups of units composing the phage reproduce severally and that the new materials are then taken up by an assembly mechanism (working after reproduction or more likely concurrently with it) that organizes them into mature particles (15); recombination would simply express the chances for the alternative forms of a given genetic unit to be brought together with those of another. This picture requires, of course, confirmation by other evidence before it can be considered as anything more than a working hypothesis. One of its features, which I find particularly appealing, is that it considers the virus, as we know it in its morphologically complex extracellular state, as a sort of resting stage, which appears to be the terminal form of a complex process of reproduction. In studying phage production, we would, then, be dealing at some point directly with events taking place beyond the geometrical and chemical limitations placed by such a highly organized structure as an active phage particle. This strengthens my confidence that phage workers—with different tools but with shared enthusiasm—are all prying into the level itself at which the mystery of biological replication is enacted.

[4] Possibly, inside lysogenic bacteria the phage is always in such form but for the occasional outbursts of phage liberation (10).

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BIOCHEMICAL STUDIES ON MULTIPLICATION OF BACTERIAL VIRUSES[1]

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THE PURPOSE of this paper is to summarize those chemical studies on virus-infected bacteria which suggest the mode of multiplication of the bacterial viruses.[2] In this survey we shall not consider the important subjects of adsorption or lysis but primarily those major synthetic steps culminating in virus formation. These steps have been studied in a system whose technical attributes permit controllable virus infection of essentially all the bacterial host cells. Various aspects of the biology and manipulation of the system have been summarized in numerous reviews.

We shall deal in the main with some even-numbered T phages, T2, T4, and T6, which parasitize *E. coli*. These tadpole shaped viruses are about 120 mμ long and 80 mμ across and contain large amounts of nucleoprotein organized within the head, which is bounded by a distinct membrane. T2 and T6, for instance, contain about 40 per cent of desoxyribose nucleic acid (DNA), and at least 55 per cent protein (1–4). There is some discussion going on as to whether these 2 components might not represent the entire phage. The absolute purity of various types of purified preparations creates a rather difficult problem.

We have recently shown (5) that when ultracentrifugally purified preparations of these viruses are found to contain phosphorus (P) other than DNA-P, these preparations still contain bacterial debris. This debris then has been shown to contain most, if not all, of the non-DNA-P. In any case well over 95 per cent of the total P is DNA-P, in contrast to the host which may contain three to five times as much ribose nucleic acid (RNA) as DNA.

When the host cells of a growing culture are infected, the cells stop multiplying. In Warburg respirometers, the increase in the rate of O_2 consumption observed in the growing culture is stopped by virus infection but respiration continues unchanged (2). This is true also on treatment of cells with virus inactivated by ultraviolet light. This simple experiment indicates that the production of respiratory enzymes has been halted; however, energy production appears to be maintained by the host's enzymes, a view which is strengthened by the findings that the RQ of the infected cell is unchanged as is the ratio of carbon burned to that assimilated. Of course maintenance of respiration alone is no measure of the maintenance and development of utilizable energy; evidence on the quantity of assimilation is essential to this conclusion.

In addition it is found that N and P are assimilated, and that nucleoprotein is being made in considerable amounts (3, 6). However, although the P entering normal cells passes through intermediates and is deposited in large measure in RNA and DNA, the P entering infected cells ends essentially in DNA, the nucleic acid characteristic of virus. This P appears there in such amounts and at a rate which suggests that it is the same P which in the uninfected cell would have appeared in the two nucleic acids. That is, there appears to be a basic shunt at some stage of P utilization which throws this P into the path of DNA synthesis. Much of this P may be isolated later in virus.

This shunt in P utilization has seemed to us to be crucial to the economy of the infected cell in establishing the parasitic relations which are being described. In line with our interest in the

[1] This research was supported in part by a grant from The Commonwealth Fund, and in part by Office of Naval Research Contract N6 ori-188, Task Order 1, NR 135-055.

[2] Some recent essays on virus multiplication have implied a rigorous separation of the levels of organization being examined by chemical and biological investigations as well as of the deductions possible from the two lines of work. The inacceptability of this viewpoint in general is especially manifest in the field of virology where it is the task of the biochemist to study genetic duplication at molecular and intermolecular levels.

precise site of the shunt, we have recently studied and demonstrated an enzymatic pathway for ribose phosphate formation from 6-phosphogluconic acid in cell-free systems (7).

It has been shown by means of radioactive P in several laboratories (3, 8, 9) that about 75 to 80 per cent of the P appearing in virus comes from the medium after infection. It has not yet been resolved whether the extra 20 to 25 per cent comes from low molecular phosphorus-containing intermediates or from host DNA.

In the course of these studies we have shown (3, 8) that the P appearing in DNA does not pass through an appreciable fraction of RNA, i.e. RNA is not a precursor of DNA in this system. Indeed the bulk of the original nucleic acid of the host, the RNA, appears to be quite inert under conditions of infection. Recent unpublished experiments of Hershey *et al.* indicate that once the virus DNA is formed, its P also does not have an appreciable turnover.

The course of DNA synthesis in *E. coli* infected with each of 2 related virus strains, e.g. T6r$^+$ and T6r, is presented in figure 1 (10). It can be noted that there is a lag of about 10 minutes before DNA synthesis begins, that synthesis in each case falls on the same curve, and that this synthesis occurs at a constant rate for most of the course of synthesis. Since intracellular virus, as will be shown later, is increasing, we can say that above initial virus:cell ratios of 4, the rate of virus DNA synthesis is independent of the numbers of intracellular virus particles.

It has also been shown that the maximal rate of virus DNA synthesis in infected cells was the same whether the infecting virus was T2r, T4r, or T6r (10). The amount of the DNA produced approaches the amount of DNA found in the virus liberated from these cells (10).

At this point, some conclusions may be formulated. The host bacterium weighs about 10^{-12} gm., the virus 10^{-15} gm. In an infected cell the respiration and quantitative assimilation pattern of P and C resembles the capabilities of the enzymes of the host. Furthermore the amounts of material synthesized imply the continued activity of these enzymes in the infected cell. Nevertheless the compounds produced are built in the main into virus, and at rates independent of the numbers of virus particles and, to a certain extent at least, independent of the kind of virus. We conclude from these considerations that the complex virus polymers are being synthesized by host enzymes and, in addition we note that these rates of synthesis of virus substances are constant, unlike most instances of bacterial growth and division which are exponential processes.

Since this suggested that intracellular virus

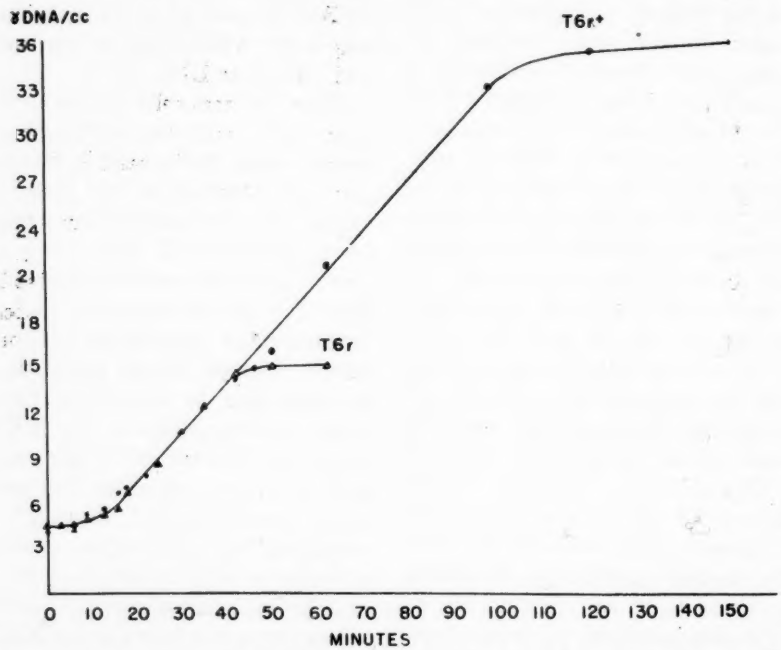

Fig. 1. A COMPARISON OF SYNTHESIS OF DNA in cells multiply infected with T6r$^+$ or T6r virus (10).

formation was also a nonexponential process, it was important to study the correlation of DNA synthesis and the formation of complete virus. After Dr. Doermann worked out the method to be described in his paper, we compared the formation of DNA and intracellular T4r virus in the same infected cells in joint experiments (11). In figure 2, it can be seen that the rates of synthesis were essentially similar, i. e. that intact virus formation is also an additive and not an exponential process. However, it should be noted that DNA synthesis does not finish off the virus —there is an additional step involving several minutes to be explored.

Protein is synthesized from the very beginning of infection (fig. 3). There is considerable synthesis, again at a constant rate, and independent of the genetic r character of the virus (10). There are many important difficult problems to be solved about this protein, one of which is the question of whether it is all virus protein. It is suggestive but not conclusive that bacterial respiratory systems are not being synthesized and Monod and Wollman (12) have shown that certain adaptive enzymes cannot be synthesized in infected cells. Fowler and Cohen (13, 14) have shown the dependence of virus synthesis on amino acids and other substances in the medium by many methods and these data as well as the serological specificity of the phages imply de novo synthesis of phage protein. These questions are also being explored by Kozloff *et al.* (15) who have shown with isotopic N that 92 per cent of virus protein N comes from the medium after infection and not from the host N existing prior to infection.

In line with the demonstration of nutritional requirements for virus synthesis, we have shown that specific amino-acid antimetabolites such as 5 methyl tryptophan among others can reversibly block virus formation (16, 17, 13). Thus a knowledge of the synthetic steps in virus formation leads to a rational chemotherapy of virus infections (11). It is not intended to minimize the technical problems of obtaining such data and developing a chemotherapy for virus diseases of man and other forms. However, it must be emphasized that a methodology for a rational chemotherapy of virus infections does exist.

From the ultraviolet absorption spectra of infected cells, it is possible to get a picture of purine and pyrimidine formation (18). The spectra for DNA, the virus, and the bacterial host, all have marked peaks at 2600 Å due to the purine and pyrimidine absorption. If we follow the increment at this wave length in suspensions of infected cells and multiply by the density, concentration factor established on DNA, we can convert this increment to DNA. If we simultaneously estimate protein-bound DNA chemically, the curves are found to be quite parallel but not coincident. These data would seem to imply that purines and pyrimidines are synthe-

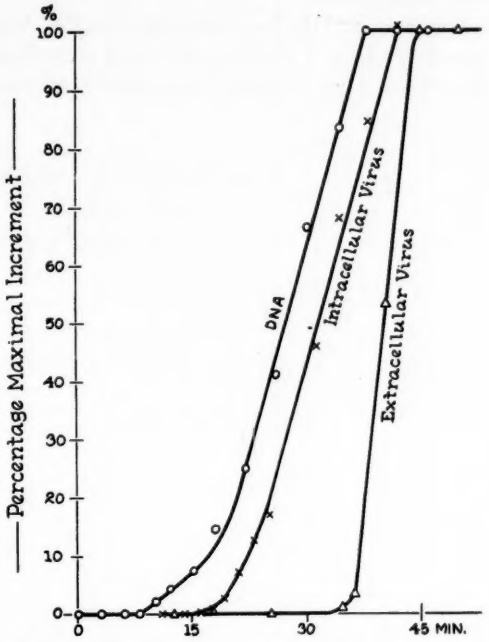

Fig. 2. THE SYNTHESIS OF DNA and intracellular T4r during the latent period of virus multiplication (11).

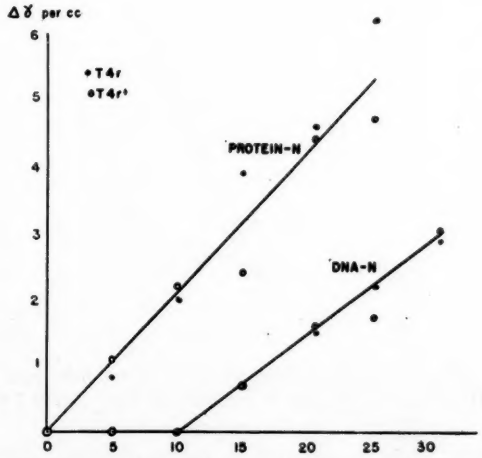

Fig. 3. A COMPARISON OF PROTEIN AND DNA synthesis in cells multiply infected with T4r$^+$ or T4r virus (10).

sized a few minutes before appearing in protein-bound DNA. Furthermore, the DNA synthesis appears to account for all the purine and pyrimidine newly formed.

Barry *et al.* (19) have recently reported that one-third of the N of virus DNA may come from host DNA. They have not yet concluded whether this N is transferred as polynucleotides, or is derived from a more deep seated turnover involving smaller fragments.

At this point we may again briefly state some conclusions. It has been shown that the synthesis of virus constituents occurs in stages, protein first, followed by purines and pyrimidines, followed by protein-bound DNA, culminating late in the latent period with the appearance of intact intracellular virus. Thus the data led us to the generalized statement that virus constituents are synthesized in stages by host enzymes according to the specifities imposed by the infecting virus particle (3, 11). This conclusion derived from studies of the metabolism of virus-infected cells is identical with a hypothesis that might be deduced from a continued inability to demonstrate metabolically active systems in isolated virus suspensions.

This conclusion leads to another curious postulate (18). The head of a T2 particle has a diameter of about 800 Å; the head, containing nucleoprotein, is surrounded by a membrane. How can we imagine specific nucleoprotein at the center of such a head, 400 Å within a mem-

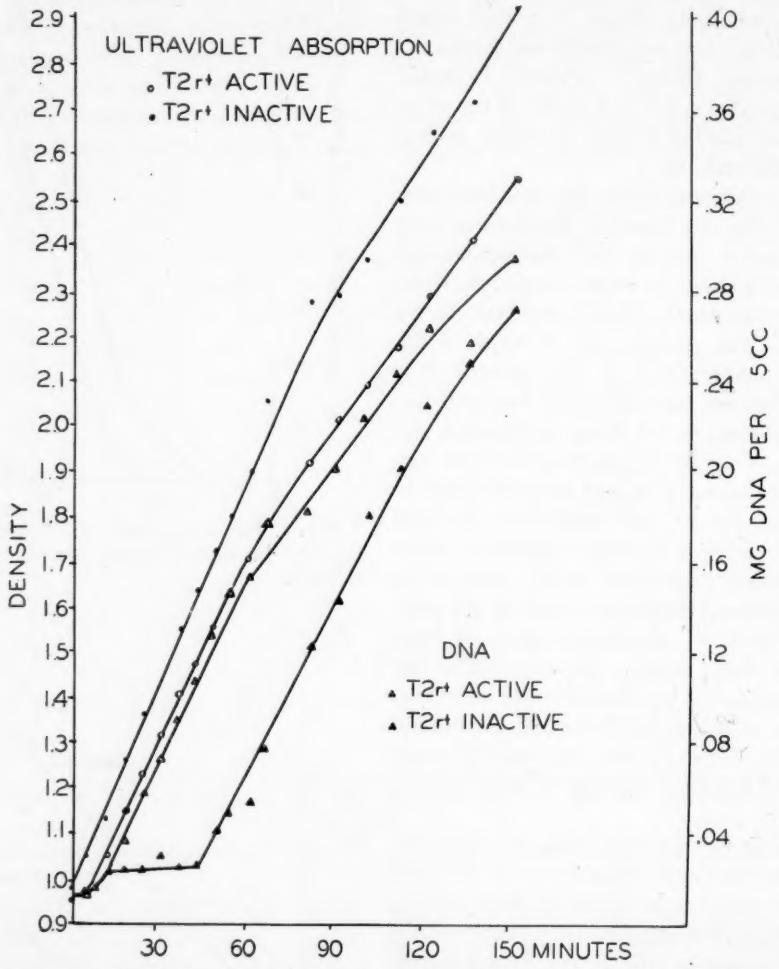

Fig. 4. A COMPARISON OF DNA synthesis and ultraviolet absorption increments at 2600 A in cells treated with active or irradiated T2r+ (virus : cell = 6.0) (18).

brane, to be synthesized by host enzymes? Since the only known chemical forces operate at maximal distances of 3 to 5 Å, it must be imagined that the initial infecting virus is disrupted when it penetrates the cell. Otherwise the internal directing pieces of the virus would be unable to come into contact with the host enzymes. That is, it is postulated that the organism as we know it in its isolated infectious state, loses its integrity as a prerequisite for duplication. Some direct chemical evidence has recently been presented that this may be so (20). The recent biological bases of this postulate and its parent discussed above will be considered extensively in the papers of Dr. Doermann and Dr. Luria.

Recently we have been studying synthesis in cells treated with virus inactivated by ultraviolet irradiation (18). Luria and Dulbecco (21) had found that several irradiated phages may initiate an infection under certain conditions when each alone is incapable of doing so. They have proposed and tested the idea that a phage contains genetic units which may be inactivated randomly by irradiation and that several such phages may reshuffle their active genetic units within the infected cell. We wished to test the hypothesis that the duplication of individual units may be an independent process, and have infected cells with partially inactivated virus under the condition wherein they would be mutually reactivated, i. e. all necessary genetic units were present in several different virus particles. DNA synthesis was followed on the assumption that the synthesis of this substance is functionally related to specific duplication.[3]

A marked lag in DNA synthesis was observed followed by synthesis at the normal rate (fig. 4). However, the absorption at 2600 Å was even

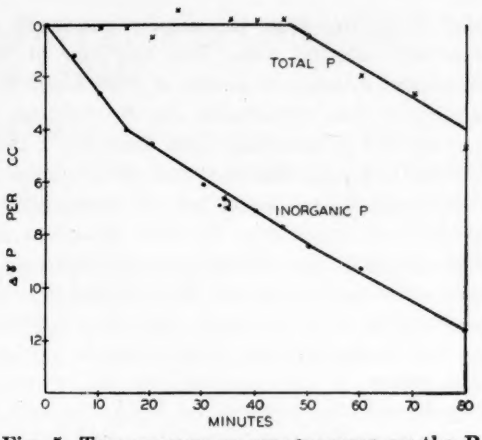

Fig. 5. THE CHANGE IN THE NATURE OF the P in the trichloroacetic acid extracts of cells treated with virus irradiated with ultraviolet light, under conditions of mutual reactivation. The cells were at a concentration of 10^9/cc.

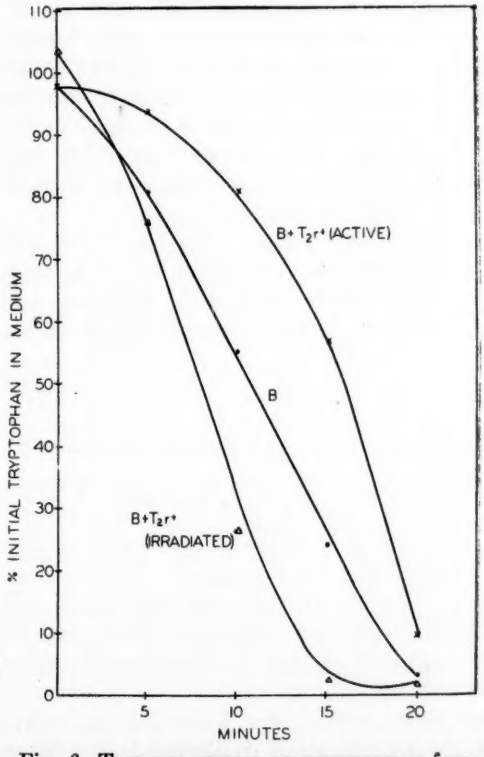

Fig. 6. THE DEPLETION OF TRYPTOPHAN from a mineral medium containing amino acids and glucose by *E. coli* infected with T2r+ or irradiated T2r+ phage, under conditions of mutual reactivation. The medium contained 10γ of each amino acid/cc., including tryptophan. The cells were at a concentration of 10^9/cc.

[3] That DNA may have a special role in determining specific genetic phenomena in general rests at present on the following evidence: *a*) the transformation phenomenon in pneumococcus and possibly other microorganisms, and *b*) the apparent correlation of DNA content and chromosome number in haploid and diploid cells. Among the bacterial viruses, the following may be added: *c*) the multiplication of radiation-sensitive units capable of going on to become virus begins approximately with the beginning of DNA synthesis in virus-infected cells (22); *d*) the time course of DNA synthesis parallels and precedes complete virus synthesis by several minutes; *e*) the period in which virus mutation is possible within the infected cell appears to correspond to the period of DNA synthesis; *f*) the amount of virus synthesized is apparently proportional to the amount of DNA produced.

slightly greater than the density increment in normally infected cells. The spectrum of the increment maintained a peak at 2600 Å and this absorption was presumably due to synthesis of purines and pyrimidines. Thus there was a total inhibition for a definite period at the level of DNA polymer synthesis, but not apparently of purine and pyrimidine. We have measured the appearance of intracellular virus by Doermann's method in this system and it was found that the appearance of intracellular virus was displaced as was the inception of DNA synthesis, i. e. the appearance of one particle per cell coincides closely with the beginning of DNA synthesis in this system. Thus a total inhibition of DNA synthesis and complete virus formation occurred for a limited reshuffling period despite the presence within the infected cell of a full complement of active genetic units. Therefore we have concluded that if DNA synthesis is essential to genetic duplication, the reproduction of genetic units requires the interdependent activity of all the undamaged genetic units. The alternative is that an increase in DNA has nothing to do with the reproduction of genetic units (18). Clearly this system may provide a critical test of the bare chemical requisites of genetic continuity.

In a study of P uptake in this mutual reactivation system we have found that there is no binding of P to protein before DNA is formed, i. e. there is no synthesis of protein-bound nucleic acid at all. As can be observed in figure 5, inorganic P was converted to organic P and these organic phosphates, possibly some early stage of DNA precursors, accumulated within the cell in an acid soluble form. Obviously the precise nature of the small amounts of these phosphates which accumulate in this system is an important problem. Although there was no net increase in protein-bound P or nucleic acid, protein synthesis proceeded nevertheless, i. e. there was an increase in trichloroacetic-acid precipitable nitrogen almost as fast as in the normally infected cell. In another approach to the same problem, Raff and Cohen have shown that the removal of tryptophan from the medium in this system prior to DNA synthesis was even faster than in normally infected cells (23) (fig. 6). We do not yet know whether this system is synthesizing virus or bacterial protein under these conditions.

Thus while normally infected cells permit a study of DNA synthesis uncomplicated by RNA synthesis, cells infected with irradiated virus may under special conditions permit the study of protein synthesis uncomplicated by any nucleic acid synthesis as well as the study of interrupted stages of DNA synthesis. Indeed the bacterial virus systems are important biochemical tools in the study of protein and nucleic acid synthesis no less than their importance in the study of the basic problem of how a virus multiplies.

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INTRACELLULAR PHAGE GROWTH AS STUDIED BY PREMATURE LYSIS[1]

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EVER since the beginnings of research on the reproduction of bacteriophage, one of the most imposing difficulties has been that the infected host cell presents a closed door to the investigator. At the beginning of the growth cycle, one or more virus particles become attached to the bacterium and after a certain length of time the cell bursts, yielding anywhere from a few to a thousand new phage particles. The purpose of this symposium is to summarize what is known concerning the events transpiring behind that closed door. From Dr. Cohen's discussion it is seen that considerable information is available concerning the chemical changes which occur within the infected cell; and Latarjet's (1) experiments with x-ray survival of phage inside infected bacteria have given some quantitative information about the intracellular picture. Yet in the absence of a method for titrating *intracellular* bacteriophage, it has been difficult to translate these results into a biological framework. My purpose in this discussion will be twofold: first, it will be to describe briefly the methods which have now been developed for estimating intracellular virus and to summarize the results which have been obtained; second it will be to present genetic data, coupled in part with intracellular assays. Similarly to the results presented by Dr. Cohen, these results will force us to the conclusion that the infecting particle must undergo some alteration prior to reproduction, and that its multiplication is not accomplished by serial fissions of the whole infecting virus particle.

With reference to the first objective, we have devised two methods of titrating the intracellular bacteriophage population. Either of these methods gives satisfactory results under the proper conditions. Due to lack of time it is impossible to describe the methods in all particulars, nor is it possible to give all the details of evidence for accepting the results which have been obtained with them. They will, however, be briefly described and compared. Both methods depend on disrupting the infected bacterium, and since we know of no methods that will do this instantaneously without inactivating the virus, they both depend also on arresting phage reproduction during a period in which the cells are broken down. The first technique, which will be referred to as the cyanide-lysis method, relies for stopping phage growth on the introduction of 0.01 M cyanide. In the presence of this inhibiting agent, the bacteria are opened up by adding a large excess of a second phage type. In our experiments T6 has been used exclusively for this purpose, and the adsorption of large numbers of this second phage causes lysis of the cells (2). In order to assay the phage whose growth we are studying in the presence of a large excess of the lysing agent T6, it is necessary to plate against the bacterial strain B/6 which is specifically and totally resistant to T6, while all the other phages which we have used form plaques when plated against B/6.

The second method, which will be called the sonic method, was devised in cooperation with Dr. T. F. Anderson in his laboratory at the Johnson Foundation of the University of Pennsylvania. This method, by contrast with the other, does not depend on cyanide to stop virus multiplication, but depends on reducing the temperature to 5° or lower during a 5-minute interval in which the infected cells are exposed to intense sonic vibrations. This treatment disrupts some 90 to 95 per cent of the infected bacteria. This method is unfortunately applicable only to those phages which show moderate resistance to sonic energy. Among the members of the T series these are T3, T7, and possibly T1 (3).

The first results which I would like to discuss

[1] Work at Oak Ridge done under contract No. W-7405-Eng-26 for the Atomic Energy Commission.

[2] The author is indebted to Dr. M. Demerec and the staff of the Department of Genetics, Carnegie Institution of Washington, for providing facilities and a stimulating environment for these experiments, and to the staff of the Biology Division, Oak Ridge National Laboratory, for material and intellectual aid in preparation of the manuscript.

are seen in figure 1, and are from an experiment done with Dr. Anderson. Its purpose was to compare the results obtained with the two methods described above. Separate aliquots of a T3-infected bacterial suspension were treated simultaneously with the two methods. From a third untreated aliquot control lysis was determined. Attention should be directed to several aspects of the results shown in the figure. It is seen that, in the ascending portions of the curves, there is close agreement in the results from the sonic disruption treatment and the cyanide-lysis treatment. The only discrepancy is found in the earliest points, where both curves fall far below the control lysis curve. This difference is due to a difficulty in technique, as is seen from the following considerations. Any infected, unlysed cell will make one plaque. In the case of the cyanide and T6-treated aliquots very nearly 100 per cent of the cells are broken down by the treatment. With the sonic treatment, however, only some 90 to 95 per cent of the cells are disrupted. From the figure it can be seen that the titer of plaque-forming particles per ml. in the early stages with sonic treatment is between 5 and 10 per cent of the infected bacterial titer. As the yield of phage per cell approaches one, the small fraction becomes negligible.

The agreement in the ascending portions of the curves can be taken to indicate that both methods give about the same intracellular phage estimate. It is furthermore evident that the two procedures are independent of each other since separate techniques of disruption have been employed and since different mechanisms were used to stop phage growth. The agreement between the results, therefore, constitutes convincing evidence for accepting these methods as adequate for titrating the intracellular bacteriophage population.

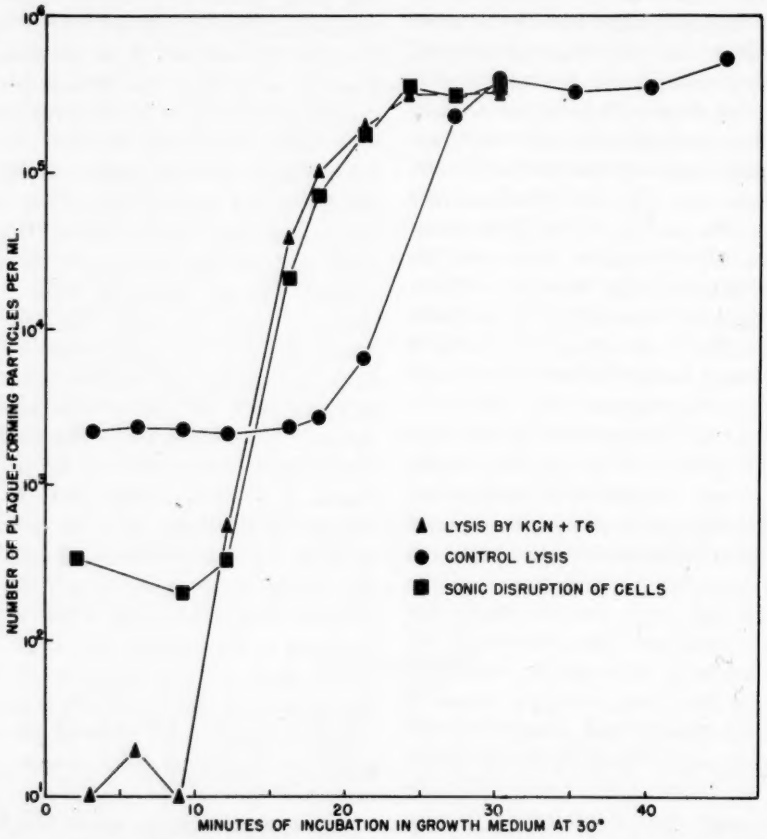
NUMBER OF PLAQUE-FORMING PARTICLES PER ML.
MINUTES OF INCUBATION IN GROWTH MEDIUM AT 30°
LYSIS BY KCN + T6
CONTROL LYSIS
SONIC DISRUPTION OF CELLS

Fig. 1. Comparison of cyanide-lysis procedure with sonic method for estimation of intracellular bacteriophage.

Results very similar to those obtained with T3 were found when the intracellular development

of T4 was followed by the cyanide-lysis technique. The curves were similar in shape, but spread out over the longer latent period of T4. Again none of the original infecting particles were recovered, even when the multiplicity of infection was in excess of 5 phage particles per cell.

The question which naturally follows here is how the results of intracellular titrations fit with the intracellular chemistry studies which were presented by Dr. Cohen. He has already answered this question, and I wish only to recall to your attention the striking parallel in the accumulation of newly synthesized desoxyribose nucleic acid in the cell and the subsequent increase in mature phage particles.

A little more detail may be added to the results of the intracellular titrations by investigating the whole cultures are plated out. When this was done with bacteria infected with 5 to 10 T2H particles per cell, the results shown in figure 2 were obtained. The average yield of phage per cell in the 5 experiments ranged from 30 to 60, and the individual bursts were plotted as percentage of the average in a given experiment. The average yields per cell from those allowed to lyse normally was around 300. It is seen that there is a wide range, percentagewise, in the cells which were disrupted ahead of their normal lysis time, indicating that the cells are not uniform in the time at which the first phage particle appears in them. The spread found is similar to that obtained from normally lysing T1-infected cells by Delbrück (4) and T2H-infected cells by Hershey and Rotman (5).

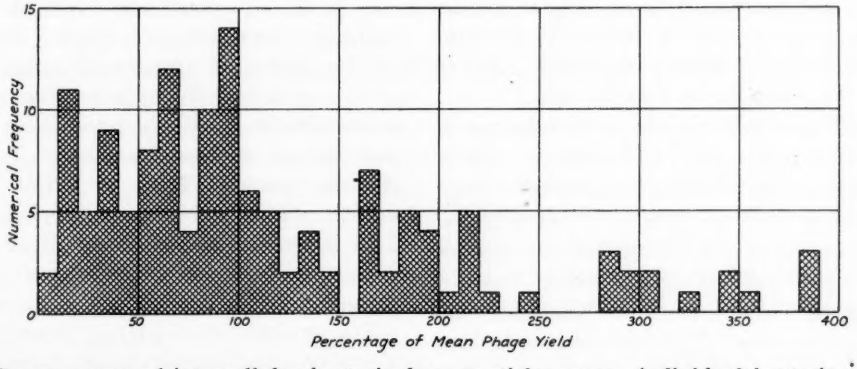

Fig. 2. DISTRIBUTION of intracellular bacteriophage particles among individual bacteria as found by cyanide-lysis procedure.

phage present in individual bacterial cells. Since the experiments of Delbrück (4) published five years ago it has been known that there is a wide range in the yield from individual cells which lyse normally. It seemed worthwhile, therefore, to adapt the cyanide-lysis procedure to titrating the phage within single bacterial cells. The technique depends on diluting the infected bacteria to a point where single drops will have a small probability of containing an infected bacterium. From this highly diluted culture single drops are dispensed into many individual tubes at some stage before one wants to induce lysis. The majority of the cultures which contain an infected bacterium under these conditions will contain only one. The cultures are incubated at the desired growth temperature until the time of lysis induction. They are then placed in an ice bath, and cyanide and T6 are added quickly. After a suitable period for lysis induction, the

TABLE 1. OCCURRENCE OF GENETIC RECOMBINANTS IN THE CROSS T2Hr_{13} x T2Hh DURING THE LATENT PERIOD

EXPER. NO.	TIME OF CYANIDE TREATMENT	NO. PHAGE PER CELL	TOTAL RECOMBINANTS OBSERVED		PERCENT RECOMBINANTS
			rh	r^+h^+	
1	20	2.3	6	4	2.0
2	20	6.0	49	48	2.2
1	25	36	2	6	1.6
3	27	67	45	36	2.4
2	29	102	18	16	2.3
1	30	113	11	8	3.0
1	Control burst	288	31	16	3.0
2	Control burst	516	72	59	3.5
3	Control burst	365	15	12	2.3

The number of bursts obtained in these experiments is slightly lower than would be expected on the basis of the number of infected

bacteria known to be present. This is to be expected, however, if a few of the infected cells do not yet contain any mature phage particles at the time of induced lysis. The fair agreement between the number of bursts and the expected number indicates clearly that the treatment used for inducing lysis is obtaining phage from all of the cells and not merely from a few which might be sensitive to the lytic procedure.

From the results so far presented, the major conclusion appears to be that the infecting particles, whether they be one or many per cell, are not recoverable as such, and that they have undergone some change that prevents them from forming a plaque when the cell is disrupted. This is in agreement with the much earlier experiments of Delbrück and Luria (6) in which they infected cells simultaneously with T1 and T2 (α and γ). Under these conditions, T2 completely suppresses the multiplication of T1. Furthermore, when the yields from these cells were examined, not even the infecting particles of T1 were found.

I would like now to turn to some of the genetic experiments which shed a little more light on the problem of intracellular reproduction of bacterial viruses. From the data so far presented one might still believe that the intracellular multiplication is accomplished through binary fission of the infecting virus particle. The non-exponential nature of the curves of intracellular virus increase could be explained by assuming some limiting synthetic reaction, and the early disappearance of the infecting particles might be due to a temporarily irreversible attachment to the bacterial components. The genetic experiments, however, furnish convincing evidence against the hypothesis of binary fission.

The basic experiments in this regard have been done by Hershey and Rotman (5). They infected bacteria simultaneously with two types of T2H which differed from each other in two differentiable hereditary loci. The yield from such bacteria consisted of the two parental genotypes, but in addition, two new types which were identified as the other two combinations of the genetic markers. When the yields from individual bacteria were analyzed, the two genetic recombinant types were found in equal proportion and furthermore the recombinants occurred in statistically the same proportion in each of the individual cells. In other words, there were no yields containing unusually large clones of the recombinant types. Even the mere fact that recombination of genetic material does occur would be difficult to explain on a binary fission hypothesis. However, assuming such a hypothesis, and admitting the possibility that whole phage particles could transfer genetic properties, one must still account for the non-clonal distribution of the recombinants. The amendment would, therefore, have to be added to the hypothesis that recombination occurs only in the later stages of multiplication.

We have attempted to answer the question whether any of the first completed phage particles may be of the recombinant types. A cross was made after the technique of Hershey and Rotman (5), but instead of allowing the latent period to go to completion, it was interrupted at several points with the cyanide-lysis technique. The cross used was one of those described by Hershey and Rotman, namely T2Hr_{13} × T2Hh. When the cells are allowed to lyse normally, this cross gives 2 to 3 per cent recombinants. This particular cross with low recombination was selected in preference to high recombination crosses for the following reason: on the binary fission hypothesis the absence of clones would more definitely preclude the early occurrence of recombinants than in cases where a high percentage of recombination occurs.

The results of the experiment (table 1) show that even when there is a small number of phage particles per cell, the percentage of recombinants is about as high as is found when cells are allowed to lyse normally. On the hypothesis of binary fission, these early recombinants would necessarily grow into clones. Hershey and Rotman's evidence is unequivocal that no clones are found. One must therefore conclude that the phages of the T-even series do not multiply by binary fission, and further that the first completed phage particles are not themselves the parents of those viruses which appear later within the same cell.

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